Regulation of Atypical PKCs

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Abstract

Protein phosphorylation plays an essential role in a diverse array of signalling cascades and regulates many cellular processes. Protein kinase Cs (PKCs) constitute one of the families of kinases involved in phosphorylating substrates on serine or threonine residues. These kinases were initially identified as being receptors for tumour promoters (phorbol esters) and the conditions required to activate different isoforms determine the subgroup classification of the 10 isoforms.

Classical PKCs (α,βI, βII and γ) depend upon Ca^{2+} and lipids (DAG, PS-phospholipids.) Novel PKCs (δ,ε,η, μ and θ) are insensitive to Ca^{2+} but are activated by lipids, DAG and phospholipids. The atypical PKCs (ζ,ι and λ.) differ greatly. These proteins are insensitive to Ca^{2+} and phorbol ester binding. The lack of knowledge on the control of the atypical PKCs has made the role of the atypical PKCs more elusive. Nevertheless, PKC ζ has been implicated in cell growth and differentiation. Moreover, PKC ζ is thought to be involved in a plethora of signal transduction pathways, including the Ras and MEK/MAPK pathways. The related atypical PKC ι may be involved in UV induced apoptosis and insulin signalling.

The aims of this thesis are to define the control and biological role of the atypical PKCs - primarily focusing on PKC ζ. As one approach, the project attempted to create a knockout mouse. This would help define a biological end point and therefore permit elucidation of the inputs. This study led to the identification of a pseudogene and its origin is described.

As a second approach to investigate PKC ζ control, various direct paths were followed - ranging from searching for potential binding proteins and cellular localisation, to analysis of activation by lipids and phosphorylation. These studies have provided evidence for the dynamic control of PKC ζ (and PKC ι) through a kinase cascade involving the lipid kinase PI3-kinase, the lipid responsive PDK1 and finally phosphorylation of PKC ζ at a site defined as threonine 410. The operation of this pathway and its influence on PKC ζ autophosphorylation (*in vivo*) and activity (*in vitro*) are presented.

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Abbreviations

aPKC	atypical PKC
ATP	Adenosine 5'-trisphosphate
βARK	β-Adrenergic receptor kinase
bp	base pair
BSA	Bovine serum albumin
C.elegans	*Caenorhabditis elegans*
cAMP	Adenosine 3',5'-cyclic monophosphate
cPKC	classical PKC
cpm	counts per minute
DAG	Diacylglycerol
DAPI	4,6-diamino-2-phenylindole
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra-acetic acid
EGF	Epidermal growth factor
EGTA	Ethylene glycol-bis(β-aminoethyl ether)N,N.N'N' tetra-acetic acid
FCS	Foetal calf serum
FISH	Fluorescence *In Situ* Hybridisation
GST	Glutathione S-transferase
GTP	Guanosine 5'-triphosphate
IPTG	Isopropyl-β-D-thiogalactopyranoside
kD	Kilodalton
Ksr	Kinase suppressor of Ras
LTP	Long term potentiation
LY 294002	2-(4-morpholinyl)-8-phenyl-4H-1 benzopyran-4-one
M	Molar
MAPK	Mitogen activated protein kinase
mRNA	Messenger ribonucleic acid
NGF	Nerve growth factor
NLS	Nuclear localisation signal
nPKC	novel PKC
OA	Okadaic acid
OD	Optical density
ORF	Open reading frame
PA	Phosphatidic acid

PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PC	Phosphatidylcholine
PDBU	Phorbol-12-13-dibutyrate
PDGF	Platelet derived growth factor
PDK1	PIP_3-dependent kinase 1
PH	Pleckstrin homology (domain)
$PI(3,4)P_2$	Phosphatidylinositol (3,4) bisphosphate
$PI(3,4,5)P_3$	Phosphatidylinositol (3,4,5) trisphosphate
PI3K	Phosphatidylinositol 3'-kinase
PKA	Protein kinase A (cAMP-dependent kinase)
PKB	Protein kinase B (Rac or Akt)
PKC	Protein kinase C
PKD	Protein kinase D
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PLD	Phospholipase D
PP1	Protein phosphatase 1
PP2A	Protein phosphatase 2A
PRK	Protein kinase C-related kinase
PS	Phosphatidylserine
PSS	Pseudosubstrate site
RACK	Receptor for activated PKCs
Rho	Ras homology protein
S.cerevisiae	*Saccharomyces cerevisiae*
SDS	Sodium dodecyl sulphate
TBS	Tris (hydroxymethyl) amino methane buffered saline
TNF	Tumour necrosis factor
TPA	12-O-tetradecanoylphorbol-13-acetate
v/v	volume/volume
w/v	weight/volume

Chapter 1

1.1 General concepts in signalling pathways

Our understanding and enjoyment of life arises due to interpretation of complex sensory inputs:- visual images, thermal changes, noise, touch, smells. All data is processed and analysed in a highly controlled manner to elicit an appropriate response. Given a stimulus, we can respond. On the cellular level, the same phenomenon occurs. Extracellular stimuli, for example growth factors, hormones, neurotransmitters, temperature, light or chemical gradients affect cells by activating or inhibiting trans-membrane receptor coupled signalling systems which in turn mediate the production of second messengers and/or secondary responses. Second messengers proceed to activate or inhibit the activities of various cellular control proteins, including protein kinases and phosphatases. The consequential activation of distinct intracellular signalling pathways can then effect different cytoplasmic machineries or elicit a specific nuclear response, resulting in gene expression. Proliferation, differentiation or modification of the cell morphology to name but a few responses may then occur.

1.2 Signalling Cell Surface Receptors

There are several classes of signalling cell surface receptors. Receptors differ in their mechanism of transducing extracellular signals. Ligand-induced receptor activation may elicit tyrosine phosphorylation of cytoplasmic proteins, directly, via receptors with an intracellular tyrosine kinase domain or indirectly, if the receptor has no enzymatic activity, by association of non-receptor tyrosine kinases to the trans-membrane receptor, as is seen for the cytokine or haematopoetic agonist receptor. Alternatively, signalling may occur through protein Ser/Thr kinase receptors, the first to be identified in mammals was the receptor for activins, in the transforming growth factor, TGF β, superfamily (Mathews and Vale, 1991). Phosphorylation independent transfer of extracellular stimuli can occur through G proteins, which are seven transmembrane receptors with a specific and well characterised structure (Dixon et al., 1986; Maguire et al., 1976; Northup et al., 1980). G protein receptors are coupled to three membrane associated subunits (α,β,γ) (Gutkind, 1998; Leurs et al., 1998). Signal propagation requires GTP, where the guanine nucleotide binds specifically to the α subunit. G protein receptors can integrate inputs from several stimuli, for example, several hormones can

elicit cAMP release via Gs (Liu and Northup, 1998). G protein mediated signalling results in signal amplification and specific activation of PKCs by G protein-mediated pathways, as will be outlined below.

A few pathways exist that are not directly channelled through cell surface receptors (Evans, 1988). Lipid soluble ligands e.g. steroid hormones, readily diffuse into cells and interact with cytoplasmic or nuclear receptors. Growth factors may also elicit their responses by internalisation of growth factor bound receptors, which enter the endosomal pathway. Signalling cascades have been shown to be initiated in endosomal pathways and may play a role in mitogenic pathways (Beguinot et al., 1984; Smythe, 1996; Smythe and Warren, 1991). Ligands are not the only stimuli triggering the activation of signalling pathways. Others include stress alterations in membrane structure or oxidative radical species activating signalling components (Finkel, 1998).

Cell surface receptors consist of an extracellular domain, which binds ligands; a transmembrane domain and an intracellular domain, which determines how the receptor mediates intracellular signalling pathways. The membrane spanning region was found to be more than just a passive lipid anchor. For cErbB2, if Val 659 (a transmembrane amino acid) is converted to a glutamic acid, the receptor is oncogenic (Garnier et al., 1994). Furthermore, the seven transmembrane region of G proteins undergoes a shift in structure on stimulation, first discovered in the crystal structure of rhodopsin, mediating the effects of light activation (Henderson et al., 1990).

The initial event in the activation of cell surface receptors involves ligand binding to the extracellular domain. Ligands are often dimeric (e.g. platelet derived growth factor, PDGF A and B chains are joined by disulphide bridges), which specifies binding to dimerised receptors (Lemmon et al., 1997). One ligand may bind exclusively to a single receptor type, for example, ligands activating tumour necrosis factor (TNF) family receptors (with the exception of lymphotoxin α) or a large diversity of ligands can bind a single receptor, exemplified by the antigen receptors. Ligand binding induces either direct conformational changes of the receptor (transformation), resulting in receptor activation (for example, steroid hormones, which can then bind DNA directly), or receptor dimerisation or oligomerisation (Lemmon and Schlessinger, 1998) This causes autophosphorylation within the intracellular domain resulting in receptor activation. For receptor tyrosine kinases, autophosphorylation exposes either the catalytic site (as depicted by the insulin receptor activation from structural data (Hubbard et al., 1994) or

binding sites which then enable recruitment of cytosolic components. Downstream effector binding and more specifically, crucial protein-protein interactions via specific modules organise cytosolic proteins in the proximity of stimulated receptors, enabling signal transduction (Heldin et al., 1998).

Specific binding modules have been identified (Pawson and Scott, 1997). For receptor tyrosine kinases, cytosolic components bind either SH2 or PTB domains (specifically recognising phospho-tyrosine residues (Pawson and Gish, 1992). SH2 (src homology region 2) domains were first discovered in Src (Pawson and Gish, 1992; Songyang et al., 1993). The regulatory domain of phosphatidylinositol 3-kinase, PI3K, p85, binds via an SH2 domain to the C-terminal to the phospho-tyrosine residue (YpXXM) of the PDGF receptor (Escobedo et al., 1991; Klippel et al., 1992). SH2 domains are found in several different proteins, for example, enzymes, PLC γ, Ras GAP and SH-PTP2 (Welham et al., 1994), or transcription factors, signal transducers and activators of transcription, STATs (Hibi and Hirano, 1998), or other adaptor proteins, for example, Grb 2 or Nck. Alternatively, cytosolic proteins bind to a slightly larger (by 86 amino acids) phospho-tyrosine binding (PTB) domain (Kavanaugh et al., 1995; Kavanaugh and Williams, 1994). The PTB recognition sequence is N-terminal of the phospho-tyrosine residue, NXXYp. PTB domains are found in adaptor proteins, for example, Shc (Cutler et al., 1993) or in the insulin receptor substrate binding protein (IRS1) (Gustafson et al., 1995). Other structural domains important in receptor recruitment include SH3 domains (a proline-rich binding motif, found in several SH2 receptor tyrosine kinase-binding proteins) and pleckstrin homology (PH) domains, which confer the ability to bind phosphoinositol lipids (Haslam et al., 1993; Lemmon et al., 1995). For TNF receptor-mediated signalling, adaptor proteins are required to link the TNFR 1 and 2 (via TRADD and TRAF 1 and 2 respectively), binding by death domains (Baker and Reddy, 1998; Hsu et al., 1996).

How Ser/Thr kinase receptors transduce TGF β signals is unclear. However, recent evidence has deciphered more of the downstream pathway. TGF β receptor family comprises three classes, types I, II and III, for example, betaglycan (Lopez-Casillas et al., 1991). Genetic approaches in *Drosophila* and *C.elegans* have resulted in the characterisation of Mad (mothers against dpp, decapentaplegic) and Smad 2-4 (Newfeld et al., 1996; Raftery et al., 1995; Sekelsky et al., 1995) and more recently, vertebrate homologues, Smad 1-6 (Derynck and Zhang, 1996). There are three groups of Smads:- those that bind directly and are a substrate for TGF β receptors (Chen et al.,

1996; Kretzschmar et al., 1997; Zhang et al., 1997); those that associate with receptor activated Smads, and proteins that inhibit Smads, anti-Smads, for example Smad 6 or 7 (Imamura et al., 1997; Nakao et al., 1997). Smad 2 and 3 bind the TGF β receptor directly and subsequent C-terminal association and phosphorylation of Smad 4 results in its nuclear translocation and activation of transcription (Derynck et al., 1998; Kretzschmar and Massague, 1998; Liu et al., 1997). TGF β mediated pathways regulate growth and cell cycle progression.

Receptors are themselves regulated and switched off by internalisation and downregulation (Mellman, 1996) or dephosphorylation, by receptor associated phosphatases. This was initially discovered in *Drosophila* for receptor tyrosine kinase regulation (corkscrew and torso (Perkins et al., 1996) or more recently, SHP (Lanier, 1998; Myers et al., 1998; Roach et al., 1998; Stein et al., 1998; Yu et al., 1998). Phosphatases are thought to associate with plant (*Arabidopsis*) Ser/Thr kinase receptors (Stone et al., 1994), but this has yet to be established for vertebrate TGF β receptors.

Direct recruitment of cytosolic components to activated receptors, results in the activation of certain responses. Lipid metabolism may be stimulated to elicit second messengers, for example calcium and $InsP_3$, mediated by PLC γ or $PI(3,4,5)P_3$, via PI3K. Alternatively, adaptor proteins will then recruit other signalling molecules, for example Sos binding to Ras to initiate activation of the MAPK cascade and gene expression. Specific signalling pathways involved in PKC activation will be discussed below.

1.3 Eukaryotic Protein Kinases

Signal transduction is a generalised concept and encompasses several signalling pathways. The complexity and need for high degrees of regulation arises due to cross-talk between signalling molecules and kinases. It is the delicate balance between kinase and phosphatase activity which can modulate the cell's response. Eukaryotic protein kinases are a large superfamily of homologous proteins. Predictions made from sequencing the mammalian genome are that around 1 or 2% of all genes encode protein kinases. Post-translational modifications of proteins by phosphorylation has been well established as a principal mechanism of regulation of cellular functions in eukaryotes (Hunter, 1995). Phosphorylation (commonly, by transfer of γ-phosphate groups from ATP onto hydroxyl groups) of serine, threonine or tyrosine residues in the substrate protein trigger conformational

changes which alter the properties of the protein leading to the physiological response appropriate to the particular agonist. Therefore understanding the role and regulation of kinases and phosphatases (their specific effectors, agonists or substrates) will lead to the unravelling of many cellular processes.

1.4 PKC introduction

Protein kinase C (PKC) was originally identified in 1977 as a cyclic nucleotide independent protein kinase (PKM), which was proteolytically activated by a calcium sensitive protease. When assayed in a cell free system, PKC was found to be activated by calcium in a phospholipid-dependent manner (Inoue et al., 1977; Takai et al., 1977). Moreover, at physiologically low concentrations of calcium (10^{-7} M range), PKC required diacylglycerol (DAG) in addition to phospholipids for activation (Kishimoto et al., 1980; Takai et al., 1979).

Studies carried out in platelets initially demonstrated that activation by thrombin, collagen or platelet activation factor, PAF (Kawahara et al., 1980; Sano et al., 1983) resulted in a concomitant phosphorylation of two proteins, a 20kD and 40kD protein, together with the subsequent release of platelet granules (Ieyasu et al., 1982). The 40kD protein was found to be an *in vitro* PKC substrate. The disappearance of inositol phospholipids from the membrane (Bell and Majerus, 1980; Kawahara et al., 1980; Rittenhouse, 1979) was always linked to the release of platelet granules and phosphorylation of a 40kD protein, suggesting that PKC physiologically mediated the response. Moreover, on stimulation, platelets rapidly and transiently produced DAG (which has a 1-stearoyl-2-arachidonyl backbone (Holub et al., 1970). Synthetic DAG added to platelets induced phosphorylation of the 40kD protein (used as an indicator of PKC activity) and it was shown that there was no change in intracellular calcium (using quin2, a calcium indicator (Rink et al., 1983). Therefore, it was concluded that PKC becomes activated in response to extracellular stimuli by transiently induced DAG. This reversible activation of PKC by DAG was a turning point in signal transduction, linking protein phosphorylation and inositol phospholipid turnover.

After this discovery, the connection was made between PKC and a synthetic DAG-mimicking compound, phorbol esters (TPA). Phorbol esters are tumour promoters. Several kinetic studies suggest they act at the cell surface membrane (Blumberg, 1980; Weinstein et al., 1979) and can mimic the action

of hormones and neurotransmitters and stimulate cell proliferation. This led to the classification of PKCs as receptors for phorbol ester (*in vivo* and *in vitro* (Castagna et al., 1982; Yamanishi et al., 1983). Phorbol esters are able to intercalate into the phospholipid bilayer for prolonged time periods, since they are only metabolised very slowly, which explains the prolonged proliferative effects on cells. Experiments using ^{3}H-PDBu (a ^{3}H- labelled phorbol derivative, less hydrophobic than TPA) demonstrated that only in the presence of phospholipids and calcium could phorbol esters bind to purified PKCs (Kikkawa et al., 1983). PDBu bound to PKC in a 1:1 relationship in the presence of phospholipids. The question of whether PDBu bound directly to PKCs or not was raised after experiments using a photoaffinity-labelled probe of phorbol esters (Delclos) was found to interact primarily with phospholipids (Delclos et al., 1983). The elucidation of the structures and cloning of the PKC family members helped to resolve this issue.

1.5 The PKC Family

PKCs are serine/ threonine protein kinases, which fall into the AGC superfamily of kinases. The AGC kinase classification includes kinases activated by cAMP (PKA), cGMP (PKG), of course DAG/phospholipids (PKC), related kinases (PKB, which was identified as being related to the A and C kinases (RACs) and will be discussed later) and kinases which phosphorylate G protein-coupled receptors (βARKs). The CaMK (family of kinases regulated by calcium and calmodulin) and CMGC (a family of cyclin-dependent kinases and MAPK) kinases are other related Ser/Thr and dual specificity kinases.

The first PKCs to be identified (PKC α, PKC β, PKC γ) were isolated from brain cDNA libraries by low stringency screens (Coussens et al., 1986; Parker et al., 1986). PKC isoforms are abundant in the brain and further screens yielded three additional PKCs - PKC δ, PKC ε, PKC ζ (Ono et al., 1987; Ono et al., 1989). Screening of cDNA libraries from other tissues has led to the identification of the other PKC isoforms, known to date - PKC η (Osada et al., 1990), PKC θ (Osada et al., 1992), PKC ι (Selbie et al., 1993), PKC λ (Akimoto et al., 1994) - and the PKC-related kinases, the PRKs (Mukai and Ono, 1994; Palmer and Parker, 1995) and PKD (Van Lint et al., 1995), PKC μ (Johannes et al., 1994). Based on their amino acid sequence similarity and enzymatic properties, the PKC isoforms have been classified into three subgroups.

The classical PKCs (cPKCs) are the best characterised, possibly due to having been identified first. This subgroup consists of isoforms PKC α, PKC βI, PKC βII and PKC γ. The β gene undergoes alternative splicing, resulting in two isoforms differing in their 50 C-terminal amino acid residues (Coussens et al., 1987). cPKCs are activated by calcium, phospholipids, phosphatidylserine (PS) and DAG (or phorbol esters experimentally).

The second subgroup is the novel PKCs (nPKCs), comprising PKC δ, PKC η, PKC ε and PKC θ. These isoforms are insensitive to calcium but are still activated by DAG or TPA in the presence of PS. The use of phorbol esters as an experimental tool to manipulate PKC function *in vivo* has greatly enhanced our understanding of the role of the cPKCs and nPKCs.

The third subgroup is the atypical PKCs (aPKCs):- PKC ζ and PKC ι (PKC λ, the murine PKC ι isoform). These are the most diverged of all PKC family members since they are insensitive to calcium and DAG, but are activated to some extent by phospholipids, for example, PS (Ono et al., 1989).

The PKC related kinases (PRKs) are similar to the aPKCs in that they are insensitive to calcium and DAG/phorbol esters but have a unique regulatory domain containing, homology regions (HR), which interact with Rho A (Amano et al., 1996; Flynn et al., 1997; Watanabe et al., 1996). PKD is also unusual due to its being activated by DAG and phorbol esters and yet it has a very different kinase domain. PKD also has a putative PH domain (Gibson et al., 1994). It is questionable as to whether PKD is a PKC family member (Rozengurt et al., 1997).

The alignment of the PKCs allows the identification of various conserved regions. The catalytic domains show a high degree of homology but the N-terminal regulatory domains are much more diverse and provide the main basis for PKC subgroup classification (Figure 1.1). Within the regulatory domain are motifs which define a distinct localisation or confer specific activation properties to the individual isotypes. The different PKC domains (conserved and variable regions) will be discussed in more detail.

1.6 C1 Domain

The C1 domain was initially identified in the classical and novel PKCs and consists of a conserved motif of cysteine and histidine residues. The C1 domains are capable of chelating zinc ions (Hubbard et al., 1991) in a zinc

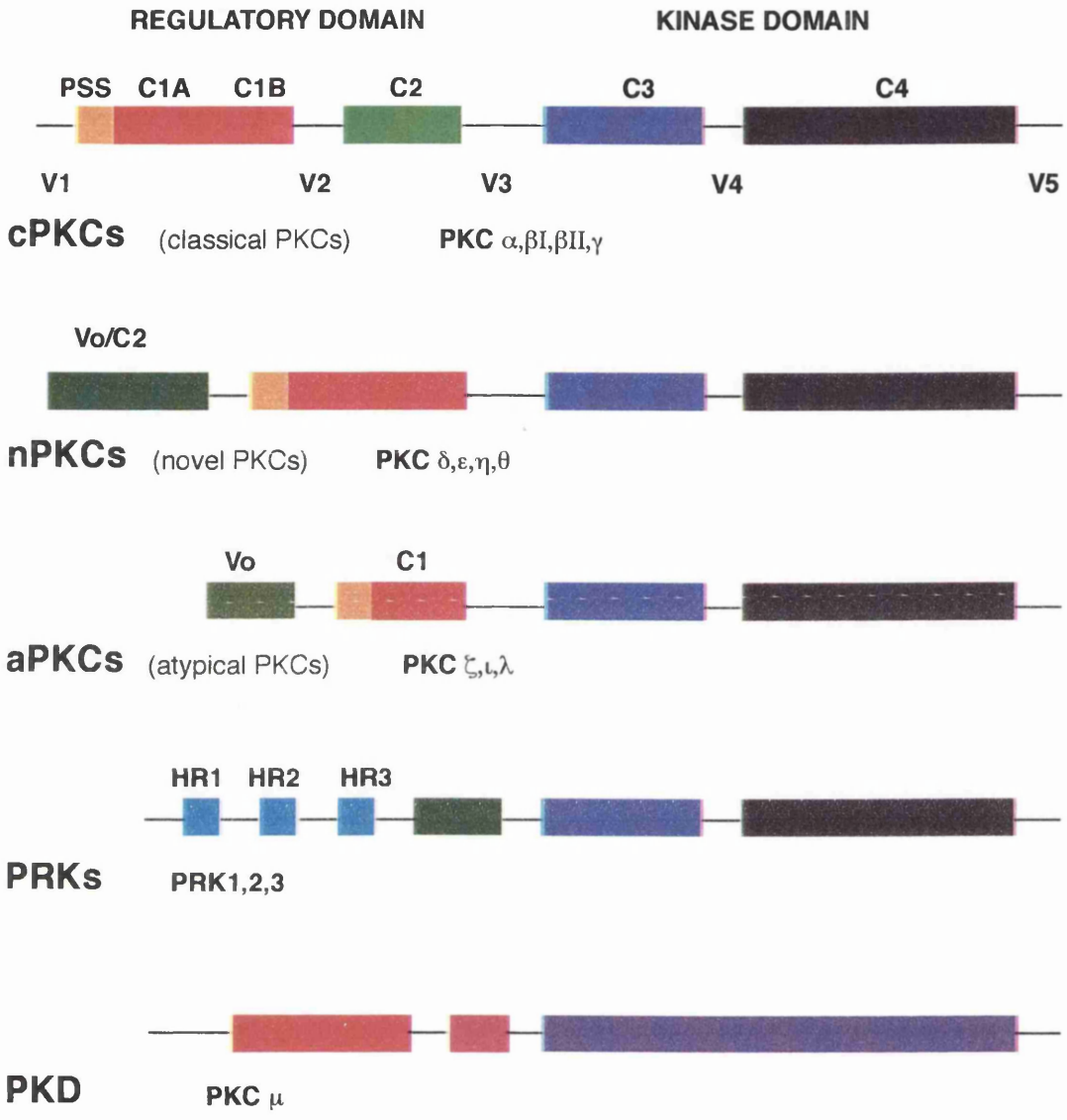


Fig 1.1
The PKC superfamily

finger structure (chelating two zinc atoms per C1 domain (Quest et al., 1992).The two metal binding sites are crucial in maintaining the fold of the structure and pulling together otherwise far apart residues (Hommel et al., 1994). The zinc finger is made up of two β sheets on the top half of the domain, which form a cavity filled with water molecules. These molecules are displaced by DAG or phorbol esters and the β strands are "unzipped". This Cys-rich motif ($HX_{12}C$-X_2CX_{10-14}-C-X_2-C-X_4-H-X_2-C-X_6-C) is duplicated in what has been classified "typical" C1 domains, to give C1A and C1B motifs (Hurley et al., 1997).

C1 regions in PKCs were found to bind phorbol esters (by mutational analysis of GST-fusion protein and crystallisation of the second zinc finger motif-PMA complex of PKC δ (Zhang et al., 1995). The C1A and B domains of PKC γ bind phorbol esters with similar affinities (Quest et al., 1994) However, *in vivo* there is a difference in affinity of the C1 domains of GFP tagged PKC γ for membrane translocation (Oancea et al., 1998). Moreover, the C1B domain of PKC δ was found to bind phorbol esters much tighter than C1A (Hunn and Quest, 1997). Therefore for PKC δ, the two domains are not equivalent and the C1B domain confers increased phorbol ester binding affinity and if the domain is deleted, decreased membrane translocation is seen (Szallasi et al., 1996). There is a possibility that *in vivo*, the two C1 domains have high and low (C1B and C1A, respectively) affinities for DAG or that in the full length protein the domains are orientated in such a way as to block access to the ligand for one of the C1 subdomains. Interestingly, phorbol ester binding does not alter the overall conformation of the domain but completes the top surface of hydrophobic residues, allowing deeper penetration into the membrane (Newton, 1995).

What determines the ability to bind DAG is unclear. A single "typical" C1 domain is sufficient to bind DAG/phorbol esters, however, the zinc finger in the aPKCs (having higher similarity to the C1A domain) does not bind DAG (Ways et al., 1992). There are certain consensus sites distinct from Cys/His residues which may determine phorbol ester sensitivity (Figure 1.2). Initially, a conserved proline (at position 11) was thought to determine binding by restricting the angle between β sheets and maintaining the pocket in an open conformation. Atypical PKCs do not have two consensus residues in positions 11 (Pro) or 20 (a hydrophobic residue). Mutating the residue in position 11 to Pro does not confer phorbol ester sensitivity (Kazanietz et al., 1994). Whether mutation of the other site would be sufficient to confer phorbol ester binding is not known. Consensus residues (Pro 11, Gly 23, Gln 27) are important in

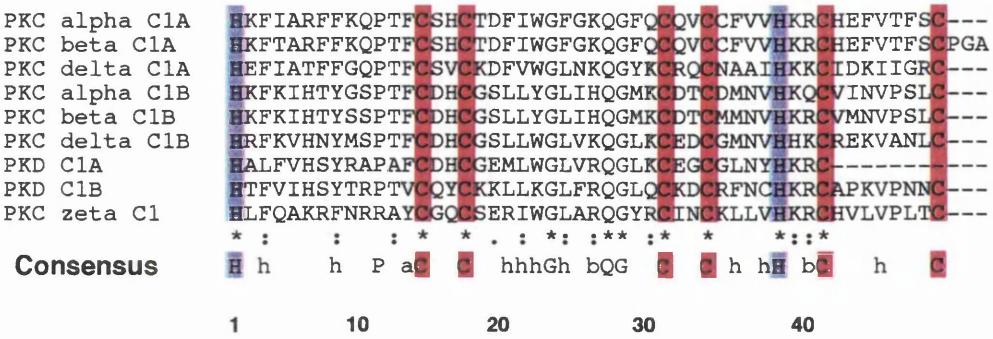


Fig 1.2
The C1 domain

Lys interacts with T497 (PKC α)

Conserved Arg residues - basic residues interact with acidic (Asp) residues in the entrance to the active site

Putative phosphorylation site (Ala) Hydrophobic interaction with Phe 350 and Met 484 (PKC α)

Fig 1.3
The Pseudosubstrate site

maintaining the unzipped structure and other residues (8,13,20,22,24 which are usually Met, Val, Leu, Ile, Phe, Tyr or Trp residues) form a hydrophobic wall around the groove and may insert into the hydrophobic core of the bilayer.

These structural sequence homologies help identify potential phorbol ester binding proteins and can be supplemented with knowledge from known C1 phorbol ester binding domains. Single or multiple copies of C1 or C1-related domains occur in other proteins- n-chimaerin (Ahmed et al., 1990), unc-13 (M-unc is the mammalian homologue), Raf, Ksr (Sundaram and Han, 1995), PKD (Valverde et al., 1994), DAG kinase and the oncoprotein Vav (Gulbins et al., 1994). N-chimaerin and PKD bind DAG and can be activated by phorbol esters.

It has been suggested that the C1 domain of the aPKCs, binds other lipids, for example, ceramide, in an analogous manner to DAG for c/n PKCs. It remains unresolved as to whether ceramide binds directly or specifically to aPKCs (Huwiler et al., 1998; Lozano et al., 1994; Muller et al., 1995). Other proteins have been found to interact with the C1 domain of aPKCs and so this domain may be essential for protein-protein interactions. Par4 binding to the zinc finger of aPKCs modulated its enzymatic activity, by inhibition of PKC ζ *in vitro* and *in vivo*, after UV-induced stimulation of par4 (Diaz-Meco et al., 1996). Conversely, PKC λ interacting protein, LIP, specifically binds to the zinc finger domain of PKC λ, stimulating activity. Thus for the aPKCs, the C1 domain does not only modulate lipid-mediated PKC activation.

The zinc finger has also been implicated in cellular localisation. DAG induced translocation to the plasma membrane has been a well established readout for PKC activation (Kraft et al., 1982). The zinc finger domain of PKC ε alone was capable of localising PKC ε to the golgi (Lehel et al., 1995). Interestingly, in the context of the regulatory domain, PKC ε is also localised at the plasma membrane.

1.7 C2 Domain

Classical PKCs were originally discovered due to their sensitivity to calcium and phospholipids and it was suggested that calcium binding may be via an EF hand domain (Parker et al., 1986). However, a unique C2 binding domain found in numerous proteins and recently in other proteins, e.g. synaptotagmin, a transmembrane protein (Sutton et al., 1995) and

phospholipase A (PLA_2) (Perisic et al., 1998), has given insight into the domain structure. There are two variations of the C2 domain, an S and P type (Essen et al., 1996).

The C2 domain has approximately 70 residues folded into loops compressing sequences at the amino and carboxyl termini in the form of a β sandwich. Calcium binding loops, CBR, come together to form a "mouth" (Grobler et al., 1996). The mouth region contains five aspartic acid residues and the carboxylate groups binding calcium. If the Asp groups are mutated to Arg residues, there is no calcium binding (Edwards and Newton, 1997). Located behind these residues are bulky hydrophobic amino acids, in particular tryptophan residues, which may act as membrane anchors, forming a highly basic surface. Interestingly, recent crystallographic (of PLC δ (Essen et al., 1996) and NMR studies (of synaptotagmin (Sutton et al., 1995) have demonstrated that C2 domains can co-ordinate two calcium ions, unlike the EF hand structure which accommodates a single calcium ion. NMR studies of PKC β C2 domain have also confirmed the multivalent calcium binding ability of PKC C2 domains (Shao et al., 1996).

It has been hypothesised that the region around the mouth of the C2 domain can also bind to the membrane (Newton and Johnson, 1998). Moreover, the C2 domain of PKC β appears to preferentially bind anionic, acidic lipids in a calcium-dependent manner (Shao et al., 1996). Another role for the C2 domain has been that of protein-protein interaction. The C2 (A and B) domains of synaptotagmin bind syntaxin and the clathrin adaptor protein, AP 2, respectively (Li et al., 1995). Receptors for activated C-kinases, RACK 1, a 36kDa homologue of the β subunit of G proteins, compete with peptide sequences of the C2 domain to bind to the calcium binding region for PKC βII (Ron et al., 1995). C2 derived peptides were found to act as specific inhibitors of hormone induced translocation and function of C2-containing PKC β isozymes.

The novel PKCs are not regulated by calcium (Sossin and Schwartz, 1993), even though residues that maintain the fold of the β sandwich are present (Newton, 1995). Recent solution of the crystal structure of the Vo domain of PKC δ (Pappa et al., 1998) shows that the nPKCs contain a C2-like domain (in the P type topology). Whilst being unable to chelate calcium ions (due to only possessing one of five conserved aspartic acid residues in the "mouth" region and having different conformations of the CBR loops), GAP43, a

neuronal substrate of PKC δ, has been shown to bind to this region in a calcium sensitive manner (Dekker and Parker, 1997).

It has recently been suggested that the basic face in synaptotagmin (specifically Lys residues) mediates inositol lipid binding (IP_4) (Irvine and Cullen, 1996). However, the basic sheet of PKC βII was found to have no effect on the membrane interaction when mutated to Ala residues (Edwards and Newton, 1997). The C2 domain clearly plays a multifunctional role and does not uniquely bind calcium.

1.8 Pseudosubstrate Region

PKC was originally identified as becoming more active on proteolysis. This suggests it is under some form of structural autoinhibition. The pseudosubstrate (PSS) region was defined as being an autoinhibitory domain, primarily modelled on the role of the regulatory domain of PKA binding in the catalytic cleft (Taylor et al., 1990). The PSS sequence is identical to that of a potential serine/threonine substrate phosphorylation site, however, an alanine residue replaces the predicted Ser/Thr phosphorylation site (House and Kemp, 1987). Therefore, this sequence has an affinity for the catalytic active site and without ATP catalysed phosphorylation, the dissociation of this sequence from the active site is energetically unfavourable. Mutation of this region results in effector-independent activity (Pears et al., 1990; Ueda et al., 1996) and so provided experimental evidence that the PSS site blocks access of substrate to the active site. Newton's group demonstrated that the PSS is more exposed to proteases after allosteric activation by DAG and PS (Orr et al., 1992; Orr and Newton, 1994), further suggesting the positioning of the PSS in the catalytic cleft in the inactive enzyme. The PSS sites of all PKCs are slightly different and this may confer substrate specificity to the unique isoforms (Nishikawa et al., 1997). However, they all have certain conserved basic residues (Figure 1.3).

Precisely what results in the release of PSS autoinhibition is unknown. Lipid and allosteric effector binding to the C1 and C2 domains activate PKCs, however, crystallographic models show only localised conformational changes on ligand binding. However, the membrane itself may facilitate release from PSS autoinhibition. A peptide mimicking residues 19-36 of PKC β bound PS containing membranes (Mosior and McLaughlin, 1991). Moreover, if accessible, the PSS region may stabilise interaction of PKC with the membrane. Lipid binding has also been postulated as affecting substrate

conformation and so influence the ability of being phosphorylated by PKC (Vinton et al., 1998). Arg-rich proteins or peptides (i.e., protamine sulphate) can also release the PSS from the active site in a cofactor independent manner (Leventhal and Bertics, 1991). Thus protein-protein interaction may also be important in pulling the PSS away from the catalytic domain. PSS binding proteins have also been implicated in cellular localisation. p62 which binds aPKCs, was found to localise them to an endosomal compartment (Sanchez et al., 1998).

1.9 Kinase domain

The catalytic regions of eukaryotic protein kinases are highly conserved (Hanks et al., 1988). The kinase domain consists of 250-300 amino acids and is subdivided into twelve conserved subdomains which fold into a catalytic core structure. Kinase domains are required to undertake three separate roles essential for substrate phosphorylation. Firstly, binding ATP (or GTP) and ensuring correct orientation of the phosphate donor with divalent cations (Mg^{2+} or Mn^{2+}), secondly, correct orientation and binding of substrate (protein or peptide) in the active site and thirdly, catalysing γ-phosphate transfer from ATP to the acceptor hydroxyl residue (a serine, threonine or tyrosine residue) of the substrate.

The general structure of the catalytic domain has been based on crystal structures of certain kinases. The characteristic features were mapped on cAMP-dependent kinase (PKA) and crystallisation of the PKA catalytic domain allowed 3D structural analysis of the basic kinase core (Knighton et al., 1991). The catalytic core consists of two lobes (one larger in size) separated by a catalytic cleft, the active site (Taylor et al., 1990). For PKA, the two lobes confer specific properties. The smaller amino-terminal lobe (subdomains I-IV) is primarily involved in anchoring and orientating nucleotides and the larger lobe is responsible for substrate interaction and phosphotransfer. The PKA catalytic domain was also crystallised with, PKI, a synthetic peptide which mimics the autoinhibitory role of the regulatory domain by blocking substrate accessibility to the active site (Knighton et al., 1991; Zheng et al., 1993).

For the PKC family, the kinase domain lies in the C-terminal half of the molecule. The mechanism of autoinhibition of PKCs is believed to be similar to PKA. Clusters of acidic residues on the entrance of the active site act as an electrostatic gate, maintaining the basic PSS residues in the active site.

However, unlike PKA, the kinase domain can only be separated on agonist-induced proteolysis (for example, on calcium or TPA stimulation or caspases for PKC δ (Denning et al., 1998) in the hinge region (V3 domain).

The kinase domains of all the PKCs are shown in Figure 1.4 (with PKA as a comparison). Kinase domains have certain invariant residues and consensus sequences throughout the superfamily (Hanks and Hunter, 1995; Taylor and Radzio-Andzelm, 1994). These are believed to be essential for function and implicate the enzyme in its role as a protein kinase. Various specific features will be discussed below. Residues believed to be essential for catalysis are listed below, mainly with reference to PKA.

ATP binding (Gly-X-Gly-XX-Gly-X-Val)
The first glycine (Gly 50 in PKA) binds the ribose moiety and the second Gly binds near the terminal phosphate (Sternberg and Taylor, 1984). Residues surrounding the Gly residues form hydrogen bonds with ATP to stabilise the interaction or form a hydrophobic pocket enclosing the adenosine ring. These include the surrounding Leu and an invariant Val residue. Lys 72 is invariant and is found 17 amino acids away from the last conserved Gly residue. It is essential for enzymatic activity by anchoring ATP and mediating phospho-transfer. If this site is mutated to another residue, i.e. to Met in PKC α (Pears and Parker, 1991) or to Arg in PKC δ (Li et al., 1995) or to Trp in PKC ζ (Diaz-Meco et al., 1993), a kinase inactive enzyme is produced. Asp 166 is invariant and is located in a DLKPEN consensus sequence. Asp 166 is the catalytic base accepting a proton from the attacking substrate hydroxyl and subsequently, in line phosphotransfer can occur. The neighbouring Lys residue, K168, stabilises phosphotransfer by neutralising the negative charge of γ-phosphate during the reaction. The invariant asparagine residue (N171) stabilises Asp 166 and assists in catalysis by interacting with α/β phosphates of ATP and chelating Mg^{2+}ions. The DFG motif is also a consensus site and interacts with phosphate groups of ATP in Mg^{2+} salt bridges.

Other residues are also important in maintaining the correct orientation of ATP in the active sites. An invariant glutamic acid residue (Glu 91) forms a salt bridge with Lys 72, stabilising its interaction with α and β phosphates of ATP. Moreover, residues in subdomain V anchor ATP by hydrogen bonding with the ribose or adenine ring, for example, Glu 127.

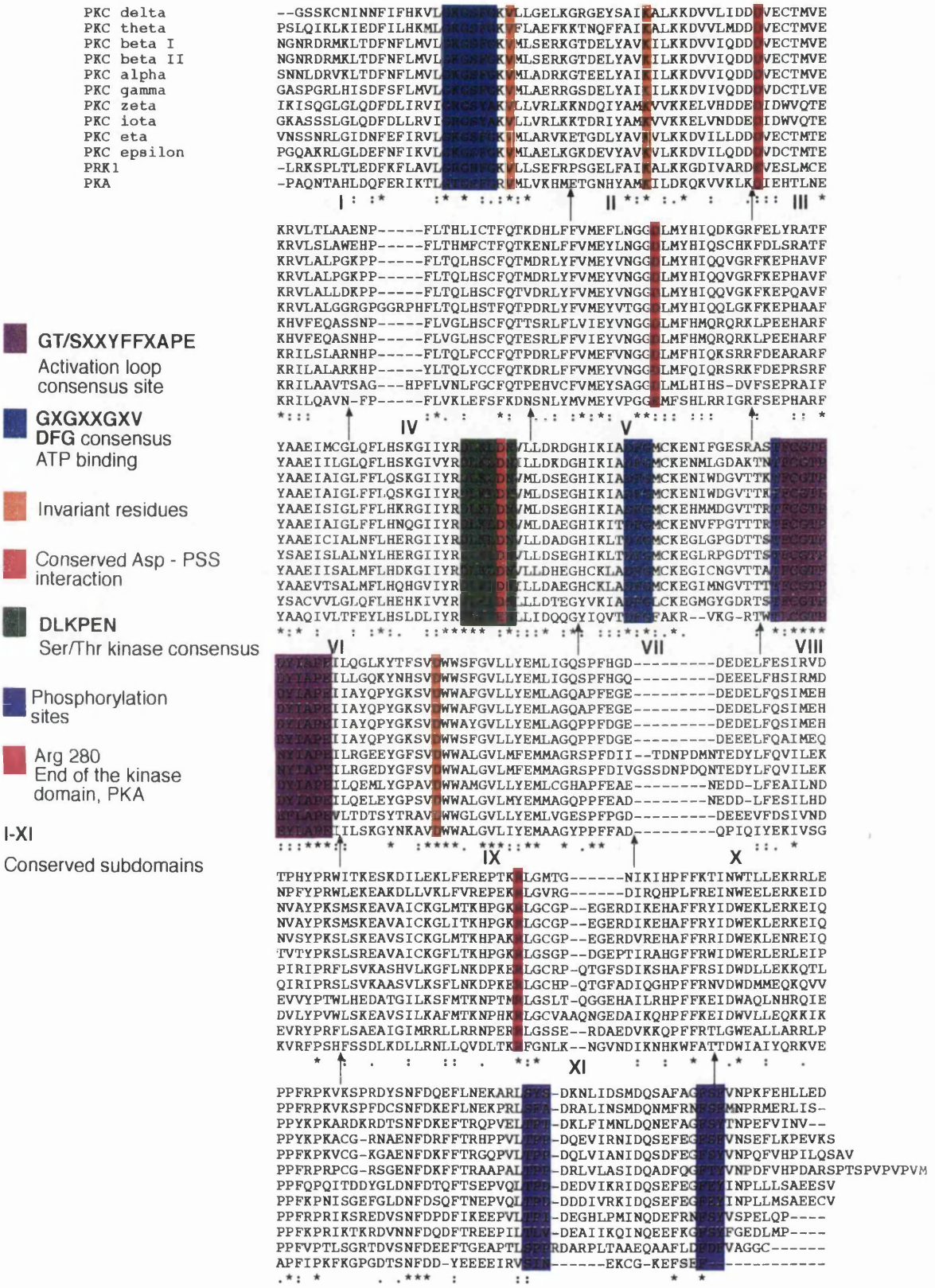


Fig 1.4
The PKC kinase domain

Activation loop site phosphorylation

The activation loop phosphorylation site is T197 in PKA, in the lip of the kinase and is discussed in more detail in section 1.12. Phosphorylation in the activation loop site results in maximal kinase activity and the conserved sequence in this region is:- **TXCGTX(E/D)YXAPE**, where X is a hydrophobic residue. The APE motif faces the cleft and helps stabilise the large lobe by ion pair interaction with residues in the XI subdomain. For PKA, Asp 220 (a highly conserved residue) in subdomain IX stabilises the catalytic loop by hydrogen bonding.

Pseudosubstrate binding

Several conserved aspartic acid residues are predicted to interact with the PSS sequence to maintain its positioning in the active site. A glutamic acid residue, Glu 127 in subdomain V, forms an ion pair with the Arg residue of the substrate consensus sequence (Arg-Arg-X-Ser-hydrophobic residue for PKA), thus stabilising substrate or PSS-kinase binding. Hydrophobic residues (residues 235-239 in PKA) in subdomain IX interact with inhibitory peptides/ sequences.

End of the Kinase domain

The most poorly conserved region of the kinase domain are subdomains X and XI and their "function" is unknown. In PKA, an invariant residue (Arg 280) defines, within 9-13 amino acids, the end of the kinase domain,

1.10 V5 Domain

The V5 domain has been implicated in playing a role in modulating kinase activity and by determining specific subcellular localisation of PKC. This region contains two phosphorylation sites in PKC α (S657 and T638). F.Bornancin demonstrated that phosphorylation of both sites is crucial for protein stability but also in particular, phosphorylation in the S657 site controls the net "on" rate of the kinase and allows further sequential phosphorylation of the other sites (Bornancin and Parker, 1997; Bornancin and Parker, 1996). If the final C-terminal region of mammalian PKC α is removed and expressed in *S.cerevisiae,* the protein was found to be inactive. Whether this correlates with protein instability and insolubility is unclear (Riedel et al., 1993). Alternatively, it has been suggested that phosphorylation of the C-terminal region may increase the affinity of the C2 domain for calcium, because PKC βI and PKC βII have different affinities for calcium. Moreover, phosphorylation at the S660 site in PKC βII resulted in a 10-fold

increase in affinity for calcium (Edwards and Newton, 1997). The crystal structure of PKA showed the C-terminal site (until residue 300) on the surface of the kinase domain. Thus there may be a direct interaction (Taylor et al., 1993).

The V5 region has been implicated in PKC cellular localisation. In U937 cells, the two alternative splice variants of PKC β show differential localisation:- PKC βI (β_2) is found on microtubules and PKC βII (β_1) in secretory granules (Kiley and Parker, 1995), possibly binding to F-actin, since this association has been seen *in vitro* (Blobe et al., 1996). A further role of the V5 domain has been in protein-protein interaction. The SXV C-terminal motif of PKC α has been shown to bind to the PDZ domain of PICK1 (Staudinger et al., 1997). PICK1 may act as a scaffold protein or act to stabilise unphosphorylated PKC α. PKC α is localised in the perinuclear region after TPA stimulation (2h) and is phosphorylated in the T250 site (T.Ng, C.Prevostel unpublished data). Since PICK1 is primarily localised in the perinuclear region (Staudinger et al., 1995), it can be postulated that PICK1 localises PKC α to the perinuclear proteasome and hence be the final signal in the PKC α degradation pathway. Therefore the V5 domain is more like a second regulatory domain and is not just a "sequence at the end" of the kinase domain.

1.11 Mechanisms of Activation

Biochemical data, detailed structural analysis and characterisation of the cellular localisation of PKC isoforms has helped to elucidate the mechanism of activation of the PKCs. Unstimulated PKC is essentially cytosolic on extraction with little intrinsic affinity for membranes. Production of DAG results in an increase of PKC affinity for the membrane as evidenced by translocation to the membrane (Kraft and Anderson, 1983; Wilson et al., 1985). This translocation "assay" was initially used as a marker of PKC activation. The purpose of membrane interaction is to open up the structure and allow accessibility to the active site. A one step model of effector activation, in this case, either calcium or DAG binding alone causing activation, does not occur (Ohmori et al., 1998). The concentration of calcium required to cause half-maximal binding or half-maximal binding of activation of PKC βII in unilamellar vesicles (40mol% PS and 5mol% DAG) are different (1μM or 40μM Ca^{2+}, respectively). Sakai *et al* demonstrated that a calcium ionophore (A23187) induced a rapid and reversible membrane translocation. However, subsequent phospholipid production induced a second and third

translocation of GFP-tagged PKC γ, resulting in a fully active kinase (Sakai et al., 1997).

PKC was originally found to be activated in a lipid-dependent manner, other factors, for example, the bilayer lipid composition must be considered in a PKC activation mechanism. Not surprisingly, in experiments used to determine which lipid increased PKC affinity for membranes the most, PKC βII had highest affinity for the naturally occurring enantiomers:- sn-1,2-phosphatidylcholine, sn-1,2-phosphatidyl-L-serine and sn-1,2-diacylglycerol (Newton and Johnson, 1998). Thus the presentation of the headgroup and bilayer composition is important. Presumably correct orientation of DAG is required to slot into the relatively immobile structure of the C1 domain. However, interestingly, translocation of PKC was found to be increased with saturated fatty acids and the kinase activity is increased by unsaturated fatty acids (Shinomura et al., 1991). Polyunsaturated DAG may result in increased spacing between lipid head groups, enhancing access of PKC to the membrane and altering the bilayer curvature, which has been found to activate PI3K (Hubner et al., 1998).

Recent evidence suggests that closer and higher affinity binding of PKC to the membrane is necessary for activation. This was demonstrated by PKC interacting with more than one PS molecule (Newton and Johnson, 1998). A larger contact of hydrophobic surfaces is required to physiologically anchor PKC at the membrane. Experimentally, using TPA may overcome certain physiological requirements for activation since it binds PKC so strongly and tightly (Oancea et al., 1998). The elucidation of the structure of the C2 domain also suggests that a larger area of the regulatory domain potentially forms hydrophobic interactions with the membrane. Oancea *et al* has recently dissected the mechanism of activation for PKC γ, by utilising various deletion mutants. If the V1 domain (Δ PSS) is removed, translocation occurs at a much faster rate, suggesting that the PSS masks the DAG binding site. Calcium is sufficient to elicit a rapid and transient response, however, DAG is required to stabilise interaction on the membrane. The suggested sequential model they propose is that calcium interacts with the C2 domain, the V1 domain subsequently binds to the membrane, exposing the C1 domain enabling DAG/membrane interaction (Oancea and Meyer, 1998).

Independent allosteric effectors alone are not sufficient energetically to remove the PSS from the catalytic cleft. For the cPKCs, as Knudson proposed for tumorigenesis, a two-hit model can be envisaged (Knudson, 1985;

Knudson, 1971). The two-hit activation model has to be somewhat revised in light of new definitions of catalytic activity. Activation of PKC by allosteric effectors alone is not sufficient to ensure maximal catalytic activity. A second event is essential for maximal activity, namely, phosphorylation (as will be discussed below). The mechanism of PKC activation is not straightforward but our understanding of activation is still strongly based on the originally identified cPKCs. Of course, defining where and what encompasses activation, for example, does this occur prior to membrane localisation, has yet to be clarified. Other important considerations include, how is PKC stabilised after synthesis when it is in an inactive state prior to membrane translocation? AKAP79 has been suggested as binding PKC α, when this occurs is unclear. Other PKC-binding proteins have been identified that are found to bind under conditions of serum starvation, for example SRBC (*sdr* (serum deprivation response)-related gene product that binds to c-kinases (Izumi et al., 1997), or to inactive forms of PKCs, putative RICKs (Mochly-Rosen and Gordon, 1998). The role these proteins play in mediating PKC stability is unknown. What role do other proteins, chaperones or scaffold proteins, for example syndecans, which may localisation PKCs to focal adhesions and membrane assembly (Woods and Couchman, 1998), play in pre-activation and membrane localisation of PKCs? GFP-PKC γ membrane translocation occurred at a slower rate at room temperature. However, no evidence was found to identify the mechanism of translocation. TPA-induced translocation is not blocked by cytoskeletal inhibitors (cytochalasin D, which inhibits actin polymerisation and colchicine, microtubule assembly). There is also no need for "energy" dependent motor proteins (Sakai et al., 1997). TPA-induced translocation can also occur without intrinsic kinase activity, since ATP binding site mutants and staurosporine treatment do not inhibit membrane translocation. The role RACKs play in PKC activation is unresolved (Mochly-Rosen et al., 1991), since they were found to bind PKC βII in the perinuclear region (Ron et al., 1995)

Does the model of cPKC activation (Figure 1.5) apply to the novel and atypical PKCs? Only the nPKCs are activated by DAG and all are insensitive to calcium levels directly. The C2-like domain structure of PKC δ in the Vo region implies that potentially the same hydrophobic surface is exposed in the C2 domain and will help PKC-membrane interactions. However, the atypical PKCs do not bind DAG and do not have a C2-like domain in the Vo region. Therefore, if the atypical PKCs are to overcome autoinhibition of the catalytic site, other events must occur. Either the Vo domain has different lipid binding sites - the "atypical" C1 domain may bind different lipids (ceramide,

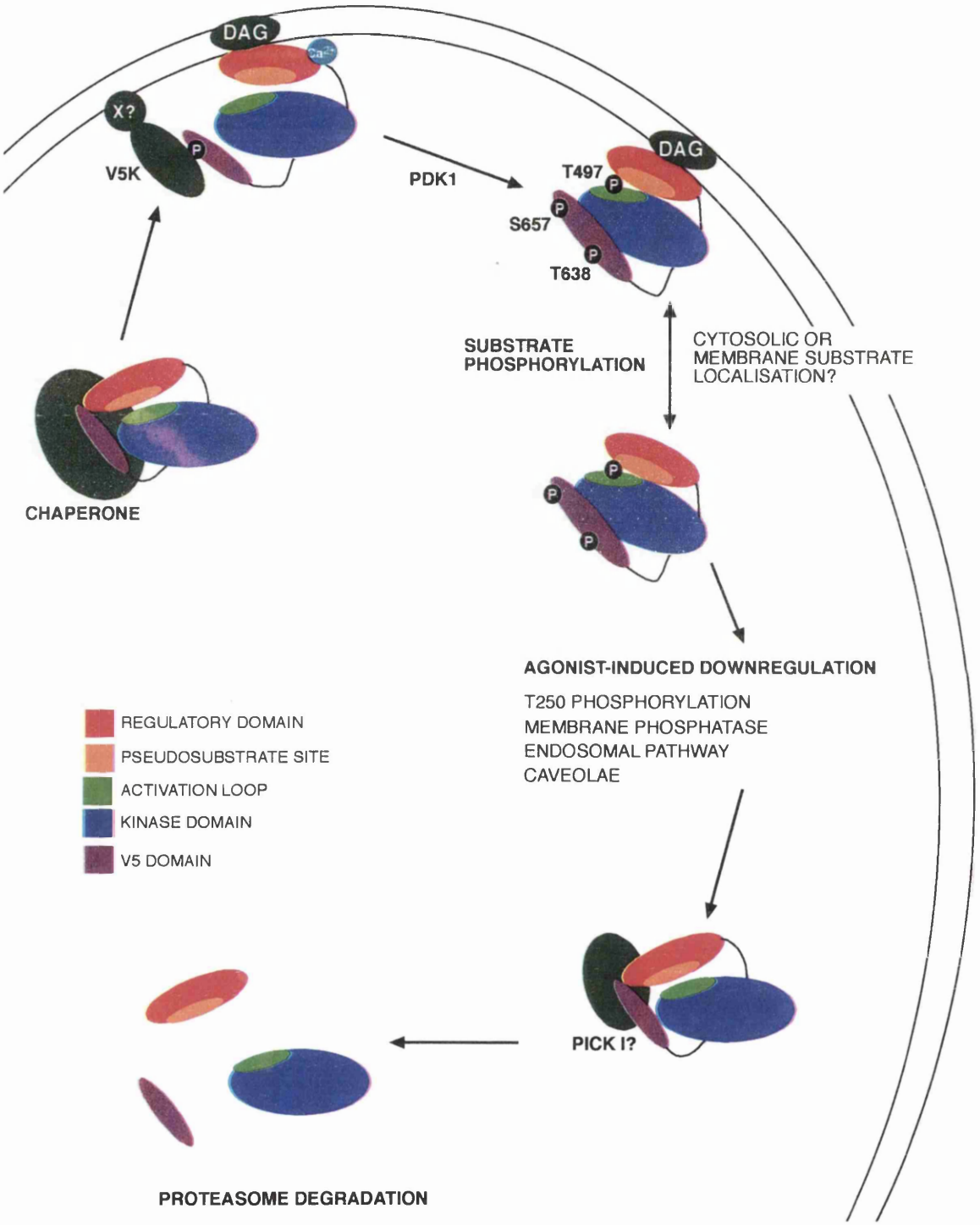


Fig 1.5
The "Life Cycle" of PKC α

phosphatidic acid or $PI(3,4,5)P_3$) or protein-protein interaction has to occur prior to the possibility of exposure of the catalytic site. Whether atypical PKCs do require membrane interaction for full activation is still unsure.

Therefore, even though the c,n,a PKCs are all fundamentally kept in an inactive conformation by binding of the PSS in the active site, different mechanisms of activation may be required to relieve this autoinhibition.

1.12 Phosphorylation

Allosteric effector regulation enables PKCs to become activated and have an accessible active site, able to phosphorylate substrates. However, further post-translational modifications are required for complete catalytic activity, namely phosphorylation.

Borner *et al* discovered several forms of PKC α in breast cancer cells (Borner et al., 1989). Pulse-labelling experiments revealed that PKC α was synthesised as a primary translation product of 74kD (a 74kD protein was produced on *in vitro* translation of the poly(A)+ RNA of PKC α) which was chased into a 77 and 80 kD "mature" form of the protein. These experiments implied that PKC α underwent post-translational modification, which was identified as being due to phosphorylation (incubation with a Ser/Thr protein phosphatases, PP1, 2A or PAP, dephosphorylated the 77kD form to 74kD). Therefore, the phosphorylation state of PKC α was associated with a shift in electrophoretic mobility.

Several groups went on to analyse the phosphorylation state of various PKCs. Flint *et al* analysed the tryptic peptides of radiolabelled autophosphorylated PKC βII isolated from baculovirus by reversed-phase HPLC (Flint et al., 1990). This *in vitro* analysis recognised four peptides containing six sites of intrapeptide autophosphorylation (S16, T17, T134, T314, T 324, T 634, T 641). Later work identified the *in vivo* phosphorylation sites of PKC βII in unstimulated cells by HPLC coupled to electrospray ionisation mass spectrometry and high energy collision-induced dissociation analysis (Tsutakawa et al., 1995) and mutational analysis (Keranen et al., 1995). Three sites were identified (correlating to four phospho-peptides) which were - T500, T641, S660. These sites were also mapped in PKC α (F.Bornancin, PJ Parker, unpublished results). It has been assumed that these are the three "priming sites" for cPKC. "Priming" sites imply a necessity for these sites to be occupied for optimal protein activity and stability.

The next question to be answered was what type of phosphorylation occurs - is it a trans- or autophosphorylation? Transphosphorylation is defined here as phosphorylation occurring by another kinase but could theoretically be by PKC itself in the form of homo-dimerisation. To examine autophosphorylation, various different techniques can be used. Bacterial or *in vitro* translation systems can be used where post translational modifications are limited. A second approach was used initially, where mixed micelles containing only one protein molecule per micelle (Hannun et al., 1985) demonstrated that PKC underwent autophosphorylation by an intrapeptide mechanism (Newton and Koshland, 1987). *In vitro* autophosphorylation assays (Mizuno et al., 1991) were used by several groups to identify potential autophosphorylation sites (Dutil et al., 1994; Keranen et al., 1995). An ATP binding site kinase inactive mutant can also be used to identify autophosphorylation sites, due to lacking intrinsic kinase activity (Li et al., 1995). Conversely, any sites which fail to become phosphorylated in an *in vitro* autophosphorylation assay or are phosphorylated in ATP-binding site mutants, are candidates for transphosphorylation sites (e.g. T500 in PKC βII and T497 in PKC α). This suggests that PKC phosphorylation is also controlled by other kinases.

Recent work has given insight into how phosphorylation affects catalytic activity and an understanding of PKC phosphorylation, on a molecular level. One site which appears to be trans-phosphorylated is the T497 (PKC α), T500 (PKC βII) site. Cazaubon *et al* demonstrated that this region containing T_{494} TST_{497} residues was responsible for the permissive activation of PKC α and that mutating these sites to Ala residues resulted in a loss of kinase activity (Cazaubon and Parker, 1993). A more detailed mutational study showed that T497 was the critical residue for catalytic competency (Cazaubon et al., 1994). The surrounding sites probably hydrogen bond and help stabilise the phosphorylation of the T497 site, in an analogous manner to T195 in PKA. PKC α wild-type protein is inactive in bacteria, however, if this amino acid is changed to a glutamic acid residue, where a glutamic acid (E) residue resembles the site of phosphorylated Thr more closely than an aspartic acid (D), the protein shows some activity (Cazaubon et al., 1994). Thus the T497 site is crucial for catalytic competency. Mutagenesis experiments of PKC βII, where a negative charge was introduced into the activation loop, demonstrated that this site is essential for catalytic activity (Orr and Newton, 1994).

T497 (for PKC α and T500 for PKC βII) falls into a region identified as an activation loop. Elucidation of the crystal structures of unrelated kinases (MAPK (Zhang et al., 1994), PKA (Knighton et al., 1991), a cell cycle dependent kinase, cdk2 (De Bondt et al., 1993) and a myosin light chain kinase, twitchin (Hu et al., 1994) has revealed that phosphorylation of this threonine residue, located at the entrance to the catalytic site, is essential for activity. The activation loop occurs between conserved subdomains, VII and VIII, in the kinase domain. The crystal structure of PKA demonstrated that when the activation loop site (T197) is occupied, the protein is in an active but also more stable conformation.

The crystal structures of the other kinases differ in the orientation of their VIII subdomains. The differences in structure can be accounted for by considering the activity state of the kinase crystallised. The negative charge introduced by phosphorylation is required to correctly align residues for catalysis. More specifically for PKA, phosphorylated T197 then interacts with multiple side chains, for example Arg 165 (which is adjacent to the proposed target base catalyst (Asp 166), and T195. These electrostatic interactions stabilise the active conformation of the protein. Some of the other amino acids which interact with the phosphate, for example, Lys 189, further suggest that a phosphate is required to orientate several key residues in the active site (Taylor and Radzio-Andzelm, 1994). The important question still remains, what is the PKC kinase responsible for phosphorylating the activation loop site?

The following table identifies phosphorylation sites which have been either mapped or postulated as being essential due to the mapping or mutational studies of PKC α or PKC βII (Table 1.1).

Table 1.1
"Priming" phosphorylation sites in other PKC isoforms, based upon PKC α and PKC βII sites

PKC isoform	*species*	*Activation loop site (TFCGTP)*	*"FSY" site*	*"TP" site*
PKC α	human	T497	S657 (FSY)	T638 (TPP)
PKC βI	human	T500	S660 (FSY)	T641 (TPP)
PKC βII	human	T500	S661 (FSY)	T642 (TPT)
PKC γ	rat	T514	T674 (FTY)	T655 (TPP)
PKC δ	rat	T505	S662 (FSF)	S643 (SFS)
PKC δ	human	T507	S664 (FSF)	S645 (SYS)
PKC ε	human	T566	S729 (FSY)	T710 (TLV)
PKC ε	rabbit	T565	S728 (FSY)	T709 (TLV)
PKC η	rat	T513	S675 (FSY)	T656 (TPI)
PKC η (L)	human	T512	S674 (FSY)	T655 (TPI)
PKC θ	human	T538	S695 (FSF)	S676 (SFA)
PKC ζ	human	T410	E579 (FEY)	T560 (TPD)
PKC ι	human	T403	E555 (FEY)	T574 (TPD)

Are the priming phosphorylation sites the only phosphorylation events occurring in PKCs? Initial *in vitro* experiments studying PKC βII demonstrated that there were six potential autophosphorylation sites, *in vitro*. 2D phosphopeptide mapping of PKC δ-K376R (an ATP binding site mutant) revealed at least two autophosphorylation sites. One is definitely known to be S643 but the other has not been characterised, but could be S662, based on data from A.Newton. Data from our lab shows that a fourth site, T250 in PKC α, is also phosphorylated in an agonist dependent manner. Thus even though there is evidence for there being three "priming" phosphorylation sites, these are not necessarily the only important regulating phosphorylation events. Other sites may become phosphorylated on further activation; become phosphorylated to target the protein to certain compartments or perhaps act as a signal for degradation.

In addition to Ser/Thr phosphorylation, PKC δ undergoes tyrosine phosphorylation in response to various different stimuli (including hydrogen peroxide). The effects of tyrosine phosphorylation on activity are different depending on the conditions under which the experiments are carried out (Denning et al., 1996; Gschwendt et al., 1994; Li et al., 1994; Li et al., 1994; Soltoff and Toker, 1995). Which sites undergo tyrosine phosphorylation in PKC δ is unclear - sites in the N-terminus (Li et al., 1996; Szallasi et al., 1995)

but also the catalytic domain (Konishi et al., 1997) have been mapped. Some sites appear to be constitutively phosphorylated. It is possible that tyrosine phosphorylation reflects more acute or extreme conditions. The role tyrosine phosphorylation plays in regulating PKC δ or the other PKCs is unknown (Konishi et al., 1997).

1.13 What is the Role of Phosphorylation?

Phosphorylation is clearly associated with catalytic competency (activation loop site phosphorylation) and protein stability. However, phosphorylation is also crucial for localisation. Kinase activity and subsequent autophosphorylation was found to be essential for cytoplasmic localisation of PKC βII after activation. The ATP-binding site mutant K371R did not dissociate from the membrane (Feng and Hannun, 1998). Phosphorylation site mutants T641A and S660A exhibited reduced membrane dissociation. Therefore it will be intriguing to discover which phosphorylation sites are required for either membrane dissociation or targeting to specific compartments or trafficking pathways and how these events correlate with the localisation of the specific phosphorylation site kinases.

1.14 PKC dephosphorylation - a role in inactivation

Just as PKC phosphorylation plays a role in activation, so dephosphorylation has been shown to be a primary event involved in degradation and turnover of the enzyme, in particular, PKC α. PKC α becomes dephosphorylated in an agonist-dependent manner on prolonged TPA stimulation (Lee et al., 1996). It is unclear what the order or precisely where dephosphorylation occurs but it appears to occur on a membrane (Hansra et al., 1996). Dephosphorylation and subsequent degradation of PKC α requires an active kinase since expression of PKM (kinase domain of PKC α) induced dephosphorylation of PKC α (Hansra *et al* submitted). This was also shown by using PKC inhibitors (e.g. bisindolylmaleimide I) to inhibit PKC activity and thus dephosphorylation. Downregulation of PKC in *S.pombe* was shown to cause a change in vesicle trafficking which was linked to PKC activity (Goode et al., 1995). Therefore active, phosphorylated PKC α is somehow partitioned into vesicles which eventually are targeted to a compartment where dephosphorylation and subsequent degradation occurs (Liu and Heckman, 1998). If vesicle trafficking is inhibited (e.g. experiments carried out at 18°C), TPA-induced PKC α dephosphorylation is inhibited (reversibly, since shifting the temperature to 37°C results in phosphorylation). More recently, the

vesicles (thought to be caveolae) and pathway taken for PKC α degradation has been elucidated (Prevostel *et al* submitted, see Figure 1.5). PKC α is dephosphorylated and is localised in a perinuclear compartment 2h after TPA treatment and is possibly directed to the proteasome, where final degradation can occur. It will be interesting to confirm the pathway and identify when all the sites are dephosphorylated and if certain sites are also important for the specific localisation of PKC α. For example, T250 site phosphorylation does not occur immediately so this site may not in fact be a site monitoring activation but may target PKC α in a stimulant-dependent manner.

1.15 Signalling Pathways involved in the Activation of PKCs

The most extensively defined PKC activators are for the classical and novel PKCs, where activation, as visualised by translocation, occurs on DAG and/ or calcium stimulation. The initial mechanism of DAG production was due to agonist induced degradation of inositol lipids (Nishizuka, 1992). However, other sources of phospholipids can also be hydrolysed to produce DAG, for example, phosphatidylcholine (PC) via PLD (Liscovitch, 1996) (see Figure 1.6). Sustained PKC activation is required for responses such as cellular proliferation and differentiation (Olivier et al., 1996). Whether the state or length of time of PKC activation reflects the cellular response is as yet unclear. Transient activation of MAPK in PC12 cells results in proliferation, however, on continuous stimulation, cells differentiate (Marshall, 1995). Initial transient production of DAG from $PI(4,5)P_2$ hydrolysis occurs in response to agonist stimulation. The reaction is catalysed by phospholipase C which is activated on stimulation of several hormones, for example, bradykinin, vasopressin, via G protein linked receptors (and Gq α subunit). Several PLC isoforms have been identified. On $PI(4,5)P_2$ hydrolysis, two second messengers (DAG and inositol-1,4,5-trisphosphate, $Ins(1,4,5)P_3$) are produced. $Ins(1,4,5)P_3$ then activates calcium store receptors (or calcium release activated channels, CRACs (Scharenberg and Kinet, 1998) and calcium mobilisation is stimulated. Thus sequentially, both cPKC activators are induced. DAG is not always produced on agonist stimulation. Some DAG is ubiquitously present due to being an intermediate in *de novo* glycerolipid synthesis (Hodgkin et al., 1998).

Concentrations of DAG in cells varies. There is a local production of DAG at plasma, internal or nuclear membranes (Divecha et al., 1991). An initial, rapid rise in DAG concentration (associated with predominantly, polyunsaturated DAGs, mainly products of $PI(4,5)P_2$ hydrolysis) is followed by a slower

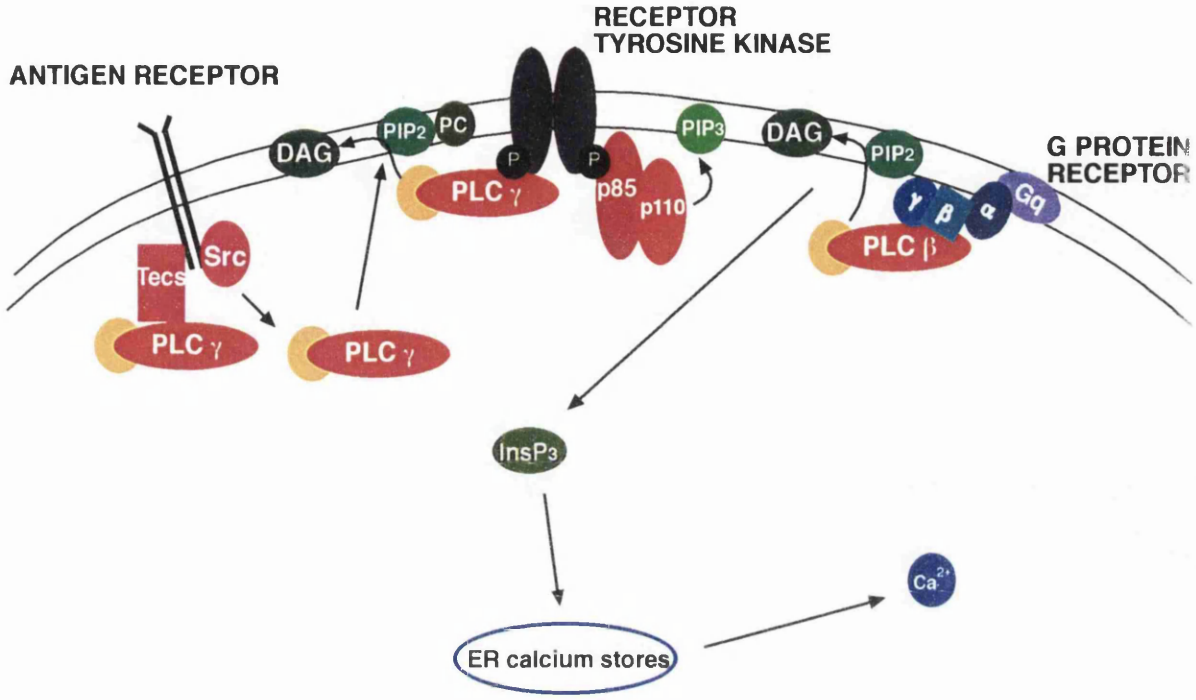


Fig 1.6
Production of cPKC activators, Ca^{2+} and DAG

accumulation and a sustained phase, lasting an hour or longer, where the concentrations of mono-unsaturated and saturated DAGs rise, occurring on the dephosphorylation of PLD generated PA (Pettitt et al., 1997; Pettitt and Wakelam, 1998). PKCs demonstrate an *in vitro* preference for polyunsaturated DAG species (Marignani et al., 1996). Mammalian cells contain 50 structurally distinct molecular species of sn-1,2-DAG (Pessin and Raben, 1989; Pettitt and Wakelam, 1993). How the different species affect activity has only recently been analysed *in vivo.* GFP tagged PKC βII trafficking from the cytosol to the membrane is a dynamic process in response to physiological signals, for example, stimulation of Gq α subunit (Feng et al., 1998). Bombesin generates polyunsaturated DAG transiently in Swiss 3T3 cells and briefly activates PKC α, PKC δ and PKC ε however, a combination of bombesin and TGF β resulted in sustained accumulation of polyunsaturated DAGs, which supported a sustained activation of PKC (Olivier et al., 1996). In thrombin stimulated platelets, activation of PKC α and PKC βII and PKC ζ correlates with a transient production of $PI(4,5)P_2$-derived polyunsaturated DAG (Baldassare et al., 1992). Interestingly, in porcine aortic endothelial (PAE) cells PLD derived DAGs do not appear to activate PKCs (Pettitt et al., 1997). Furthermore, a second phase of activation from PLD-induced DAG was not mirrored by PKC γ membrane translocation (Oancea et al., 1998). Thus, the source of lipid and lipid conformation may specifically regulate certain signalling pathways.

PC may be broken down to give DAG, mediated by PLD (see Figure 1.7). PC hydrolysis also occurs by a second pathway, namely via PLA_2. PLA_2 is a soluble enzyme which translocates to the membrane on calcium mobilisation by bradykinin, histamine, ATP or thrombin or by EGF or PDGF. PLA_2 activity results in the formation of free fatty acids and lysophospholipids, particularly, arachidonic acid, which also affects PKC activity and membrane association (Blobe et al., 1995; Shirai et al., 1998). Another potential PKC activator is phosphatidic acid (PA). This is produced on activation of PLD or by DAG-kinase catalysed phosphorylation of DAG to yield PA. The different pathways generate two species of PA- mono-unsaturated or polysaturated forms. Sphingomyelin hydrolysis stimulated by activation of TNF α leads to ceramide production (Heller and Kronke, 1994). Ceramides can stimulate phosphatases (Hannun, 1997; Hannun, 1994; Heller and Kronke, 1994) or a specific ser/thr kinase (Kolesnick and Golde, 1994), or PKCs (Huwiler et al., 1998; Limatola et al., 1994). PKC δ and PKC ε were found to translocate to the cytosol on ceramide induced apoptosis in HL60 cells (Sawai et al., 1997).

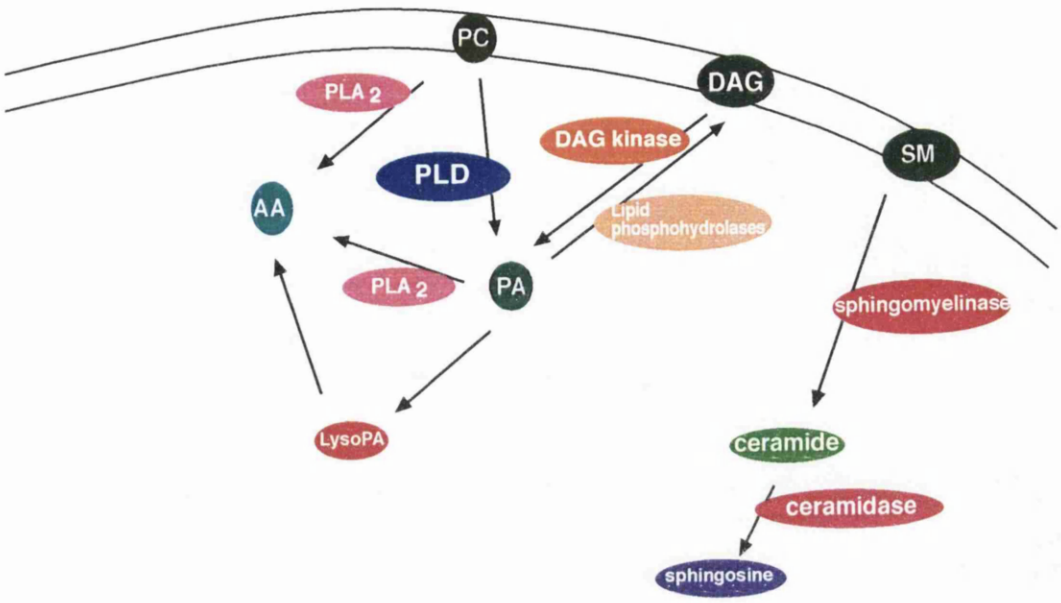


Fig 1.7
Production of PKC lipid activators

Other well-characterised lipids which can activate PKCs include $PI(3,4,5)P_3$ (Derman et al., 1997; Nakanishi et al., 1993; Toker et al., 1994). Whether this is by direct interaction of $PI(3,4,5)P_3$ with PKCs or is channelled through another protein is unclear. Phosphatidylinositol 3-kinase (PI3K) was found to make a novel series of 3-phosphorylated inositol phospholipids (Traynor-Kaplan et al., 1988; Whitman et al., 1988). A number of 3-phosphorylated inositide lipids were found to increase on growth factor stimulation. These included $PI(3,4,5)P_3$ and $PI(3,4)P_2$, even though $PI(3,4)P_2$ is believed to be a breakdown product of $PI(3,4,5)P_3$ (Hawkins et al., 1992; Stephens et al., 1993; Stephens et al., 1991). The PI3Ks are a diverse family of lipid kinases which share a homologous catalytic domain (Vanhaesebroeck et al., 1997). There are three different classes which differ in the mechanisms of regulation. Class 1a are heterodimers with adaptor/regulatory subunits (p85) bound to the catalytic subunit (p110) (Dhand et al., 1994; Gout et al., 1992; Hiles et al., 1992). Class 1a PI3Ks mediate insulin receptor tyrosine kinase signalling. Class 1b catalytic subunits are activated by binding $\beta\gamma$ subunits of G proteins (Stephens et al., 1997; Stoyanov et al., 1995). Class 2 PI3Ks cannot use $PI(4,5)P_2$ as a substrate and are resistant to wortmannin (Domin et al., 1996). Class 3 PI3Ks only use phosphatidylinositol as a substrate. $PI(3,4,5)P_3$ can also affect other receptors. $PI(3,4,5)P_3$ is only made where $PI(4,5)P_2$ exists and has been found to activate and recruit PLC γ to membranes. $PI(3,4,5)P_3$ binds directly to the PH domain of PLC γ (Falasca et al., 1998) or indirectly by binding to Btk/Tec tyrosine kinases, in B cells (Fluckiger et al., 1998). This enables PLC to be in the viscinity of its substrate, $PI(4,5)P_2$. This adds a further degree of complexity to the second messenger production pathways.

There are several hundreds of different classes of lipids in every cell. $PI(3,5)P_2$ has recently been found in yeast (Cooke et al., 1998) as a novel lipid. Thus not all potential activators of PKCs have been identified.

1.16 The Role of the Atypical PKCs

How all these ideas and models apply to the atypical PKCs has been unclear. Atypical PKCs cannot be manipulated acutely due to being unresponsive to TPA. However, information from dominant negative expression of aPKCs suggests several roles in signalling pathways, for example in mitogenic signalling (Berra et al., 1993; Dominguez et al., 1992). How our knowledge of PKC α processing can be related to the atypical PKC "life cycle" remains to be examined. Before we can understand more about the aPKCs, we need to know more about the targets and upstream controls of these proteins. Both

these aspects are tackled in this thesis. The first approach was to attempt to define a generic biological role for the aPKCs, focusing on PKC ζ, by trying to create a knockout mouse. The second approach was to look more specifically and define immediate up- and downstream controls regulating PKC ζ.

Chapter 2

2.1 Materials

2.1.1 Chemicals and Radiochemicals

National Diagnostics	Acrylagel (ultra pure),Bisacrylagel (ultra pure)
BDH Laboratory Supplies	Trichloracetic Acid, ethanol, methanol, ethanediol, Triton-X-100, Tween-20
Calbiochem	Microcystin
Pharmacia, Sweden	Glutathione 4B Sepharose, Protein A and G Sepharose
Amersham International	Radioisotopes, hyperfilm, ECL Western blotting kits, donkey anti-rabbit IgG and anti-mouse IgG coupled to horseradish peroxidase, rainbow markers
Schleicher and Schuell	Nitrocellulose membrane
Premier Brands	Milk powder (Marvel)
DAKO	FITC labelled anti-mouse antibodies
Jackson laboratories	Cy3 labelled anti-rabbit antibodies
Whatman International	Phosphocellulose P81-paper
Gibco BRL	1 kB Ladder (DNA)

All reagents used for cell culture of eukaryotic cells, including media, were obtained from Gibco BRL. Organic solvents were purchased from Heyman Ltd., England and all other chemicals were supplied by Sigma-Aldrich Company Ltd. Restriction enzymes and DNA modifying enzymes were obtained from New England Biolabs, Promega, Stratagene or Boehringer.

Kit Systems

QuiaPrep Spin Miniprep/Maxiprep Kits	**Quiagen**
Cyclist ™ Exo-Pfu DNA Sequencing Kit	**Stratagene**
QuikChange Site-Directed Mutagenesis kit	**Stratagene**
In vitro transcription-translation	**Promega**
BioRad Protein Assay Kit	**Bio-Rad Laboratories Ltd**

2.2 Methods

2.2.1 Library Screening

The phage library was titred to give $5x10^5$ - $1x10^6$ pfu/plate. On a 22x22cm dish, 30ml of top agar containing 1ml bacteria (NM 538 strain were used and were grown from an overnight culture in 0.2% maltose to an OD_{600} of 0.9 and resuspended in 0.01M $MgSO_4$ ready for phage infection) and 100µl phage (EMBL 3A λ library) was plated out and left to grow for 12-16h for discrete phage plaques to form. Hybond-N^+ membranes were placed carefully on the cooled agar surface and allowed to sit for 1min. Orientation markers were made in the agar using a needle dipped in ponceau red. Membranes were then removed and placed colony side up on 3x 3MM filter paper soaked in denaturing solution for 5min. The membrane was further placed on filters soaked in neutralising solution for 3min. This was repeated again on fresh neutralising solution. Afterwards, the membrane was washed in 2x SSC prior to drying. The process was repeated to duplicate all lifts. DNA fixation was carried out by UV crosslinking for 1 min at 0.12J in a Stratagene UV Stratalinker.

2.2.2 Membrane Hybridisation

Pre-hybridisation was carried out in a hybridisation oven at 65°C with Church's solution. Hybridisation was carried out with a labelled probe (see below) in 10ml of fresh Church's reagent at 65°C overnight. The filters were then washed at 60°C for 30min with 2xSSC, then 0.1x SSC and if necessary for a further 30min at 65°C with 0.1x SSC. Filters were dried on 3MM paper, wrapped in Saran wrap and exposed by autoradiography.

2.2.3 Random Prime Labelling of a cDNA probe

50-100µg of template DNA was incubated with 1µg random primers in a total of 15µl. The DNA was heated to 95°C for 3 min and then cooled on ice for 5min. To this mix, 3µl of 10x random prime buffer (900mM Hepes (pH6.6), 100mM $MgCl_2$), 2µl each of 0.4mM dATP,dCTP,dGTP,dTTP and 5µl [γ-^{32}P]-dCTP (10mCi/ml) were added. The reaction was started by addition of 1µl klenow (5U/µl) and allowed to run for 1h at 30°C. Unincorporated nucleotides were removed by passing the sample through a G50 spin column and the incorporation of radioactivity in the probe measured by counting, usually 5-10 x 10^8 cpm/µg DNA. Probes were heated to 95°C for 5min prior to use to ensure denaturation.

2.2.4 Southern blotting

DNA was transferred onto Hybond-N^+ nylon membranes by first denaturing the DNA in the gel by soaking in denaturing buffer (see "buffers" section) for 30min. If fragments >10kb are being analysed, depurination is required first and the gel was placed in 0.125M HCl for 15min. The gel was washed in distilled water before being submerged in neutralising buffer and incubated for 30min with gentle agitation. The gel was then transferred for 4-20h in a capillary blot (according to instructions from Amersham).

2.2.5 Double-Stranded DNA Sequencing

Sequencing of double-stranded plasmid DNA was carried out using the Cyclist Exo-Pfu DNA Sequencing kit from Stratagene according to the manufacturer's instructions. The sequencing reaction was separated on an 8% polyacrylamide gel that was run for 1.5-2h at 80 W. The gel was dried down and exposed for 15h at room temperature.

Sequencing was also carried out on the ABI fluorescence sequencing machines (ICRF central services).

2.2.6 Phage DNA purification

Phage plaques were grown in 50ml LB (Luria-Bertani) medium with 10mM $MgSO_4$ overnight. Chloroform (300µl) was added and the culture incubated for a further 5min before centrifugation at 2500rpm for 20min at 25°C. To the supernatant, 50µl DNAse and RNAse (10mg/ml stock) were added and incubated for 30min at room temperature. After centrifugation (16000rpm, 2h), the pellet was resuspended in 0.1M TRIS pH8.0 and 0.3M NaCl and treated with proteinase K. Protein was removed by phenol-chloroform extraction and phage DNA was extracted from the lower layer by ethanol precipitation.

2.2.7 Molecular Biology

General restriction digests, ethanol precipitations, phenol-chloroform extractions, and gel electrophoresis (using TAE in the running buffers) were carried out by standard protocols from the enzyme manufacturers (NEB or Promega) or as

described in Maniatis. DNA purification was carried out on a small scale by mini preps (DNA was transformed into *E.Coli* by electroporation and single colonies inoculated into media - standard Maniatis procedures) either by the alkaline lysis protocol or quiagen miniprep machine. Larger scale preparations were carried out using either the Quiagen maxiprep spin column kit or by double banding on a CsCl gradient. For large scale preparation of plasmid DNA used to transfect fibroblast cells the CsCl method was used. Two CsCl gradients were employed to guarantee an $O.D._{260}/O.D._{280}$ ratio of 1.8 to 1.9 for the resulting DNA. This was crucial for reproducible transfection results.

2.2.8 Cloning

Depending upon the strategy required, inserts were cloned into new vectors in various manners. Blunt ending was carried out by either filling in (T4 DNA polymerase) or digesting DNA with Mung bean nuclease. Incubation with 2U T4 DNA polymerase was carried out at 37°C for 30min with deoxynucleoside triphosphates, dNTPs (2mM each). Mung bean digestion was carried out at 1U (to digest 1μg DNA) for 20 min at 30°C with the standard buffer supplied with the enzyme. If a single restriction enzyme was used to cut the DNA or on double blunt-ended ligation, the vector was treated with alkaline phosphatase to reduce self-ligation. 1U phosphatase was incubated with 10-20μg restriction digested DNA for 10 min at 37°C. DNA was purified from gels by using the Quiagen gel extraction kit and used in ligations of 10μl with 1 U DNA ligase at 15°C overnight. DNA was transformed into electrocompetent bacteria using a Biorad electroporator. Electrocompetent bacteria (40 μl) were thawed on ice, added to 0.1-1μg DNA (or 1μl ligation reaction) and incubated on ice for a few minutes before electroporation in a 1mm cuvette, carried out at a pulse of 1.65kV and 25mF capacitance. SOC media (1ml) was then added to the mix and incubated at 37°C for 1h before plating out various volumes on appropriate antibiotic containing LB plates, which were incubated overnight at 37°C.

For certain vectors, blue /white selection was possible and transformed bacteria were plated out on plates containing 100nM IPTG and 2% X-Gal. Bacteria with inserts give rise to white colonies.

2.2.9 Polymerase Chain Reaction (PCR)

A standard PCR reaction mix was used, namely :-

100pmoles of each primer; 4 dNTPs all at a concentration of 1.25mM; template DNA up to 1μg (if using genomic DNA); PCR 10x amplification buffer and water to make up specific total volumes. The polymerases Vent (no additional $MgCl_2$ required) or Taq were used.

RT-PCR was carried out on various cDNA libraries (kindly given by Dr D Simmons, ICRF, Oxford) using primers described in Chapter 3. Primer annealing was at 54°C. A standard PCR protocol was used but annealing temperatures and extension times varied according to the primers and template used.

2.2.10 Mutagenesis

Various mutations were introduced at the phosphorylation sites of PKC ζ. Mutations to the cDNA were carried out using the PCR based QuikChange mutagenesis kit. The primers were designed to introduce silent mutations near the site of mutagenesis to include a new, unique restriction site. This would enable easy screening of the mutants in comparison to the wild type enzyme.
The following oligos were used:-(forward and reverse primers)

T410 E mutant:- GGCGACACAACAAGCGAATTCTGTGGAACCCCG
CGGGGTTCCACAGAATTCGCTTGTTGTGTCGCC
(Introduced **EcoRI** site)

T560 E mutant:- GAGCCCGTACAGCTCGAGCCAGATGATGAGGAC
GTCCTCATCATCTGGCTCGAGCTGTACGGGCTC
(Introduced **Xho I** site)

T560 A mutant:- GAGCCCGTACAGCTGGCGCCAGATGATGAGGA
GTCCTCATCATCTGGCGCCAGCTGTACGGGCTC
(Introduced **Nar I** site)

The mutants were made using wild-type PKC ζ in pcDNA3. The mutations were sequenced and then regions cloned into myc-tagged PKC ζ using convenient restriction sites.

2.2.11 *In vitro* transcription-translation

1μg of pseudogene DNA or vector were transcribed *in vitro* and translated in rabbit reticulocyte lysates (kit from Promega) according to the manufacturers instructions. Incubations were carried out at 30°C for 120min in the presence of ^{35}S methionine.

2.2.12 *In vivo* transfection of COS and 293 cells

COS 7 cells or HEK 293s were maintained in DMEM, supplemented with 10 % FCS in 10% CO_2 humidified air at 37°C. For transfection, cells were split to $1x10^5$ cells/ml, seeded in a 10cm plate, the day before transfection and then transfected with between 1 and 10μg DNA by the calcium phosphate method (standard protocol in Maniatis). Transfected cells were placed at 5% CO_2 overnight to encourage a precipitate to form, before changing the media the following day and switching to 10% CO_2. Proteins were allowed to express for 24-48h, depending upon which construct was transfected, before harvesting.

2.2.13 Immunoprecipitation

Cells were gently washed in cold PBS before scraping cells (whilst on ice) into (1ml for 10cm dishes) harvesting buffer (see "buffers" section). Samples were dounce homogenised and then left on ice for 10min. Samples were centrifuged at 9000 rpm, 4°C for 10min. The supernatant was then pre-cleared by incubation with protein A Sepharose (for 15min at 4°C) and centrifuged. The supernatant was added to 100μl of a 50% slurry of either protein A or G Sepharose. The Sepharose beads were pre-incubated with anti-sera (1:10 dilution) for 45 min, 4°C and then unbound antibody removed by centrifugation and washing with lysis buffer. Protein binding was allowed to occur for 1h at 4°C with rocking. Beads were collected by centrifugation and washed three times in lysis buffer. Sample buffer was added to the beads directly (100μl) if the sample was to be analysed directly by SDS-PAGE electrophoresis. If protein was to be analysed on the beads, 50% ethylene glycol was added and protein stored at -20°C. If required, protein could be eluted from beads using a suitable peptide. For the 9E10 peptide, made by the Peptide Synthesis Lab, ICRF, 10μg/ml was used, eluting protein in two batches, to ensure elution of at least 75% of protein bound to beads.

If the protein was to be used for PDK kinase assays, the beads were washed in and eluted with kinase buffer (see "buffers" section - the buffer contains no Triton-X-100).

2.2.14 ^{35}S-methionine labelling

Cells were transfected with various constructs and allowed to express for 24h (as above) before washing with media and being placed in methionine-free media (no FCS) for 1h. Cells were then placed in fresh methionine-free media containing 5% FCS and 100μCi/ml ^{35}S methionine. Dishes were harvested after a further 24h and immunoprecipitation carried out as above.

2.2.15 Total lysates

Whole cell extracts for Western analysis were washed in PBS at 4°C and samples were scraped into either directly into sample buffer (400μl for a 10cm dish) or harvesting buffer (sample buffer was added before SDS-PAGE analysis) and then sonicated (three bursts at a power setting of 20 microns).

2.2.16 Polyacrylamide Gel Electrophoresis (PAGE)

Proteins were separated according to molecular weight by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), essentially as described by Laemmli, using Hoeffer Sturdier gel apparatus. To analyse PKC ζ, 10% acrylamide running gels and 6% stacker gels were prepared. To observe smaller proteins, such as GST tagged Vo domain, 15% running gels were used. Denaturing 4 x concentrated sample buffer (4 x SB with 2M urea) was added to the protein samples (1-fold final concentration) before they were heated at 50°C for 10 min and then separated on a polyacrylamide gel. Rainbow Markers (from Amersham) were run in parallel as molecular weight standards. After electrophoresis the gels were further processed either by Western blotting or stained with Coomassie Brilliant Blue or dried down (for ^{35}S-methionine labelling and ^{32}P incorporation) and autoradiographed at -70°C with a double intensifying screen.

2.2.17 Western Blotting

Proteins were subjected to SDS-PAGE and then immobilised on nitrocellulose membrane (or PVDF) according to the semi-dry method recommended by Jancos for JC Semi-dry Electroblotters. The membranes were then incubated for 1h at room temperature or at least 12h at 4°C in PBS containing 5% (w/v) skimmed milk and 0.1% (v/v) Tween-20 or with TBS (1% BSA and 0.1%(v/v)Tween-20) if phospho-specific antibodies are used, to block non-specific protein binding sites. The primary antibody or antiserum recognising the protein of interest was diluted 1:500 - 1:3000 (as described in later chapters) and incubated with the membrane for 1h at room temperature or overnight at 4°C. After 4 washes of at least 10min in PBS (or TBS) supplemented with 0.1% Tween-20, a secondary antibody (coupled to horseradish peroxidase) was applied in a 1:5000 dilution for 45 to 60min. The membranes were washed in PBS as described above and the signal was developed using the enhanced chemiluminescence, ECL detection solution according to the manufacturer's instructions (Amersham).

2.2.18 Coomassie Staining and Destaining of SDS-PAGE Gels

Gels were incubated for 15 min in Coomassie-Brilliant Blue Buffer (0.1% w/v Coomassie Brilliant Blue, 10% acetic acid, 50% methanol) and background staining removed by several washes in Destain Buffer (10% acetic acid, 50% methanol) over a period of 12h to 16h. The gels were then dried under vacuum on a gel dryer at 80°C for 1h.

2.2.19 Antisera production

A carboxy terminal peptide corresponding to the new C-terminus of the pseudogene LLWTRL was synthesised by the Peptide Synthesis lab, ICRF. The peptide was coupled to keyhole limpet hemocyanin with glutaraldehyde (Marais and Parker, 1989) and used to immunise rabbits.

2.2.20 Immunofluorescence

Cells were plated and transfected on acid-washed coverslips. For 293 cells, coverslips were coated in 0.3mg/ml collagen solution. Coverslips were then washed in PBS before fixing the cells with 4% paraformaldehyde, for 15min (an additional 20s methanol treatment was carried out when studying microtubules).

Coverslips were gently washed in PBS between each step. Cells expressing green fluorescent protein, GFP-fusion proteins were not processed further and were mounted on slides at this stage. For other proteins, cells were next permeabilised in 0.2% Triton-X-100 for 5min, quenched in 1mg/ml sodium borohydride/PBS 10min and then blocked in 1% BSA/PBS before incubating coverslips for 1h with a primary antibody, usually diluted 1:500 in 1%BSA/PBS. The fluorescein isothiocyanate, FITC or cy3 coupled secondary antibody (1:200) was incubated for a further hour. Actin was stained with phalloidin at 0.1μg/ml for the last 20min of incubation. Coverslips were mounted in Mowiol and images viewed by fluorescence or confocal microscopy.

2.2.21 Kinase Assays

PKC ζ was assayed for activity as follows. A 40 μl assay mix was prepared containing 40mM $MgCl_2$, 20mM TRIS pH7.5, 1mg/ml PKC ζ pseudosubstrate peptide or MBP (dissolved in 0.2M Hepes and 2mM EGTA), 10mg/ml mixed brain lipids (dried down under nitrogen and sonicated in 20mM TRIS pH 7.5) and 1mM ATP, with 1μCi [γ-^{32}P]-ATP per reaction. If necessary PKC ζ was diluted into enzyme dilution buffer (20mM TRIS pH7.5, 2mM EGTA, 0.02% Triton-X-100, 0.2mM DTT) at 4°C directly before the assay. Reactions were carried out at 30°C for 5-10 min and stopped by spotting the reaction onto Whatman p81 paper and placing in 30% acetic acid. The filters were washed 3x 10min in acetic acid and then Cherenkov radiation counted.

2.2.22 PDK kinase assays

In vitro GST-tagged PDK was incubated with PKC ζ for varying times (see figure legends). PDK kinase assays were carried out in the presence of lipids, as described in (Alessi et al., 1997). Purified PDK was obtained from D. Alessi (Alessi et al., 1997) and baculoviral PKC ζ from laboratory stocks purified by P. Parker, as described previously, (Stabel et al., 1991).

2.2.23 Bacterial protein expression

pRSET (HIS tagged) or pGEX vectors were used to express various PKC ζ constructs. Expression was induced in BL21 pLys bacteria under different conditions. Cells were grown to an OD_{600} of 0.8 at 37°C and then induced with

100μM IPTG. Cultures were grown at 30°C or 25°C (for full length protein) for a further 3-5h.

2.2.24 Isolation of HIS/GST tagged proteins

GST tagged proteins were isolated according to manufacturers protocols. For HIS tagged proteins, cells were pelleted (3000rpm, 10min) and then resuspended in sonication buffer (15ml for 400ml culture.) For full length constructs, lysozyme (1mg/ml) was also added. Samples were sonicated on ice for 4x 30s and then centrifuged at 15000 rpm, 4°C for 20min. To the supernatant, 0.5ml of a 50% slurry of Ni-NTA resin was added and incubated (with rocking) for 1h at 4°C. The beads were centrifuged (1000rpm, 2min) and washed three times with HIS washing buffer. Proteins were eluted with 200mM imidazole and proteins stored at -20°C with 50% ethanediol.

2.3 Buffers

TAE 50x	2M Tris-acetate, 0.05M EDTA (pH 8.0)
TBE 10x	90 mM Tris, 90 mM boric acid, 20 mM EDTA (pH 8.0)
20x SSC	3M NaCl, 0.3M sodium citrate
Denaturing solution	1.5M NaCl, 0.5M NaOH
Neutralising Solution	1.5M NaCl, 0.5M Tris-HCl(pH 7.2), 0.001M EDTA
Church's Solution	0.5M sodium phosphate (pH 7.2), 7% SDS, 1mM EDTA (pH8.0)
PBS	8 g/l NaCl, 0.25 g/l KCl, 1.43 g/l Na_2HPO_4, 0.25 g/l KH_2PO_4
TBS	saline solution, 10mM TRIS pH 7.5

Alkaline Lysis buffers (minipreps)

Solution I	50mM glucose, 25mM Tris HCl(pH8.0),10mM EDTA
Solution II	0.2M NaOH, 1% SDS
Solution III	5M potassium acetate, 11.5% (v/v) glacial acetic acid

Harvesting (Lysis) buffer

1%(v/v)Triton-X-100, 20mM Tris/HCl (pH 7.5), 50µg/ml leupeptin, 50µg/ml aprotinin, 1mM dithiothreitol, 0.1mM phenylmethyl sulfonylfluoride

Running Buffer 10x

250 mM Tris, 192 mM glycine, 0.1% SDS

4 x Sample Buffer

250 mM Tris-HCl pH 6.8, 8% w/v SDS, 20% v/v glycerol, 0.1 M DTT, 0.001% bromophenolblue, 2M urea

Semi-dry transfer buffers

Anode I	300 mM Tris-HCl (pH 8.0), 20%methanol

Anode II	25 mM Tris-HCl (pH 8.0), 20%methanol
Cathode	25 mM Tris-HCl (pH 8.0), 40 mM 6-amino-n-hexanoic acid, 20% methanol
Kinase buffer	50mM TRIS pH 7.5, 0.1mM EGTA, 0.1% β-mercaptoethanol, 0.02% Brij 35, 50nM calyculin, 50μg/ml leupeptin/ aprotinin
Sonication buffer	50mM sodium phosphate (pH 8), 300mM NaCl, 3mM β-mercaptoethanol, 5mM benzamidine, 100μg/ml leupeptin/ aprotinin
HIS washing buffer	50mM sodium phosphate (pH 6), 300 mM NaCl, 10% glycerol, 3mM β-mercaptoethanol, 5mM imidazole

Cell Lines

COS-7	African Green Monkey Kidney Cells transformed with SV 40
HEK 293	Human embryonic kidney cell line

2.4 DNA Constructs

Table 2.1
PKC ζ and PKC ι DNA constructs generated during the course of this thesis

Construct	*Vector*	*Tag*	*Cloning details/ insert size*
PKC ζ	pcDNA3		Xba fragment (1.8kb)
PKC ζ	pEFlink	Myc	5' Nco site inserted into Xba site (oligo) - Nco/Xba (1.8kb)
kinase domain	pEFlink	Myc	Cla/Xba (1.1kb)
regulatory domain	pEFlink	Myc	Nco/Cla from myc PKC ζ (0.7kb)
REG Δ PSS	pEFlink	Myc	Cla/Xba from PKC ζ Δ PSS blunt/religate
Vo (1-99)	pEFlink	Myc	Sph/Xba from myc PKC ζ - blunt/ religate (0.32kb)
Vo + PSS (1-135)	pEFlink	Myc	Eco47III /Xba from myc PKC ζ - blunt/ religate (0.41kb)
PKC ζ A119E	pEFlink	Myc	Not/Xba into myc PKC ζ
PKC ζ Δ PSS	pEFlink	Myc	Not/Xba into myc PKC ζ
T410E	pEFlink	Myc	Nco/Bam from pcDNA3 mutant into myc PKC ζ
T410A	pEFlink	Myc	Nco/Bam from non-tagged mutant into myc PKC ζ
EE (T410E/T560E)	pEFlink	Myc	Cla/Bam T410E into T560E
T560E	pEFlink	Myc	Bam/Xba from pcDNA3 mutant into myc PKC ζ
T560A	pcDNA3		
PKC ζ	pRSET	His	PKC ζ - Xba (blunt) -Bgl II. Vector - BamHI (blunt) - Bgl II then ... Xba (blunt)/ Not Vector +PKC ζ REG - Nco (blunt)/ Not
kinase domain	pRSET	His	Cla (blunt) /EcoRI from His PKC ζ /vector - BamHI (blunt)/ EcoRI
PKC ζ	pGEX	GST	Clone PKC ζ into SL301 (Nco/Xba) pGEX - Nco/ Xho
kinase domain	pGEX	GST	Cla (blunt) /Xba pGEX - Sal (blunt)//Hind III
Vo	pGEX	GST	Sph (blunt)/Nco pGEX - Hind III (blunt) /Nco
Vo + PSS	pGEX	GST	Eco47 III/ Nco pGEX - Hind III (blunt) /Nco
REG	pEGFP-C2	GFP	Sac/Acc (0.61kb)
PKC ι REG	pEFlink	Myc	Introduce 5' Nco site - oligo - Nco fragment (0.6kb)
PKC ι	pEFlink	Myc	PflM I/ Xba into PKC ι REG (2.1kb)

Table 2.2
Pseudogene constructs (from genomic Clone 3)

Construct	*Vector*	*Tag*	*Cloning details/ insert size*
NX	bluescript		PKC ζ Ψ I - Not/Xba (1.8kb)
SX	bluescript		PKC ζ Ψ I - Sal/Xba (5kb)
X7	bluescript		PKC ζ Ψ I - 3'untranslated region - Xba (7kb)
SX	pcDNA3		PKC ζ Ψ I - Xho/Sal - Xba (5kb)
SX del	bluescript		Sal/Nhe - remove 2.5kb 5' untranslated region/ blunt- religate
SX Δ	pcDNA3		SX del - Kpn/Xba (2kb)
Ψ	pEFlink	Myc	Not/Xba into myc PKC ζ

Table 2.3
DNA constructs obtained from other sources

Construct	*Vector*	*Tag*	*Received from*
PKC ζ Δ 116-122	pCO_2		D. Schoenwasser (PP lab)
PKC ζ A119E	pCO_2		D. Schoenwasser
PKC ι T410A	pcDNA3		P. Garcia-Paramio (PP lab) (Garcia-Paramio et al., 1998)
PKC ζ	pEGFP-C3	GFP	Dr P Seechiero
PKC ζ Δ 116-122	pEGFP-C3	GFP	
Cdc2DN	pCMV		Prof. E Nigg
Cyclin B2 mutant	pCMV		
PDK	pEF link	Myc	Dr D Alessi
PDK (51-556)	pEF link	Myc	
PDK (51-404)	pEF link	Myc	
PDK (1-450)	pEF link	Myc	

Table 2.4
Cloning and Expression Vectors

Vector	*Source*
pEF link	R.Treisman (mammalian expression vector)
pGEX-KG	PP lab
pcDNA3	Invitrogen (mammalian expression vector)
pEGFP	Clontech (mammalian expression vector)
pRSET B	Invitrogen
bluescript	Stratagene
SL301 superlinker	Invitrogen

Chapter 3

3.1 Introduction

As described in the first chapter, our understanding of classical and novel PKC function has arisen mainly due to the ability to directly activate these PKCs experimentally with phorbol esters. For the aPKCs, such tools are not available. Therefore, to address the issue of the biological role of the aPKCs, primarily focusing on PKC ζ, one first approach was to create a knockout mouse. The recent ability to inactivate specific genes in mice has greatly enhanced our understanding of molecular, cellular and behavioural aspects of normal and disease processes (Rajewsky et al., 1996). Mouse knockout models can provide great insights into the role of specific proteins or add to the complexity of our understanding of genetic determination and gene redundancy. A knockout may give insights into the potential outputs or functionality of PKC ζ and therefore permit elucidation of the molecular inputs or downstream effectors.

The PKC family is an expanding group of isoforms. Most of the isoforms were isolated on screening cDNA libraries from the central nervous system. Some clones were initially isolated as partial cDNA fragments, for example, PKC ζ. Not so many PKC loci have been isolated at the genomic level and there is little information on the intron-exon boundaries of PKCs. The prime purpose of attempting to isolate genomic clones is to elucidate evolutionary origins of a gene, understand domain and gene organisation and create transgenic or knockout mice.

The strategy for creating a knockout construct was to disrupt the open reading frame of PKC ζ by disrupting the first exon. The success in screening and the clones isolated are discussed in this chapter.

3.2 Screening a Genomic Library

In attempting to create a knockout mouse, the strategy was to undertake a relatively stringent library screen with a 5' fragment of PKC ζ as a probe, hoping to bind most strongly to the 5' end of genomic PKC ζ. A mouse genomic λ gt 10 phage library (the master library was plated out in the Molecular Analysis of Mammalian Mutations laboratory, ICRF) was screened with a 300 bp N-terminal fragment of PKC ζ cDNA, which was random prime labelled with [γ-^{32}P]-dCTP. Three strongly hybridising clones were isolated after primary screening (Figure 3.1).

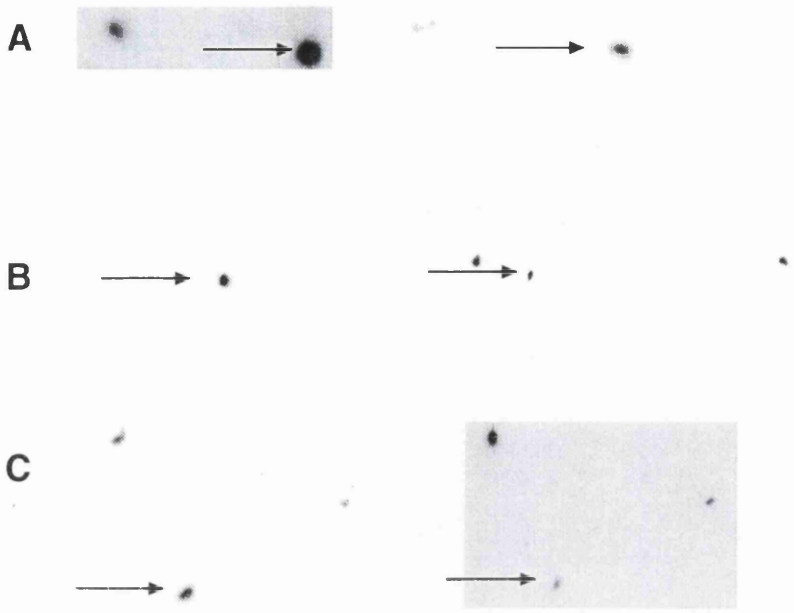


Fig 3.1
Primary screen - three genomic clones were isolated
Four filters were screened, each containing around $1x10^6$ clones. Duplicating colonies which hybridised with the probe were isolated (other spots represent a previous screening of the library). Filters on the left hand side represent primary lifts and duplicate lifts are on the right. Three clones were isolated:- A. Clone 1, B. Clone 2, C. Clone 3.

3.3 Characterisation of the Genomic Clones

The three genomic clones were purified by a further two rounds of re-plating and screening. The library was constructed by Sal I digestion and around 13kb fragments inserted into vector arms of the λEMBL3 phage. Phage DNA from the three clones was isolated (see materials and methods) and then analysed by restriction digests and Southern blotting (Figure 3.2). The phage DNA was digested with several (less frequently cutting) enzymes and combinations of enzymes. Digests were also carried out with unique enzymes, specifically cutting PKC ζ at the beginning of the ORF. The hope was to find the 5' end of coding region of PKC ζ and intron sequence.

The three clones looked identical by restriction analysis and Southern blotting and one clone, clone 3 (various fragments were subcloned into bluescript to make DNA preparations easier), was analysed further. Clone 3 contained an insert of around 13kb. From this analysis, a restriction map was constructed (Figure 3.3). Re-probing the Southern blot with a different, more specific region of PKC ζ (N-terminal 300bp) helped clarify the position of certain restriction fragments (see Figure 3.2D).

The location of certain unique restriction sites (Not I, Xho I, Pst I) in the known ORF restriction map suggested that the entire reading frame of PKC ζ had been isolated. The strongly hybridising region (SX, a bluescript construct) of Clone 3 was analysed further by sequencing. Primers were designed (200bp apart and some in reverse orientations to duplicate sequence) through the entire ORF of murine PKC ζ.

Sequencing showed the isolated clone to contain an open reading frame of 1.8kb and to be almost identical to the known murine PKC ζ cDNA (Figure 3.4). A few base pair changes occur throughout the sequence, translating into only a few changes on the amino acid level (Table 3.1). The deleted C-terminal region results in a frame shift at the protein level in the 3' untranslated region. A few hundred nucleotides at both 5' and 3' ends of the gene were sequenced. The 3' end is practically identical (there are a few base pair changes) to that of murine untranslated cDNA PKC ζ (i.e. the mRNA) and ends with an AATAAA sequence and further downstream, a poly(A) stretch.

The sequencing demonstrated that an intronless gene had been isolated, which could potentially be described as a pseudogene.

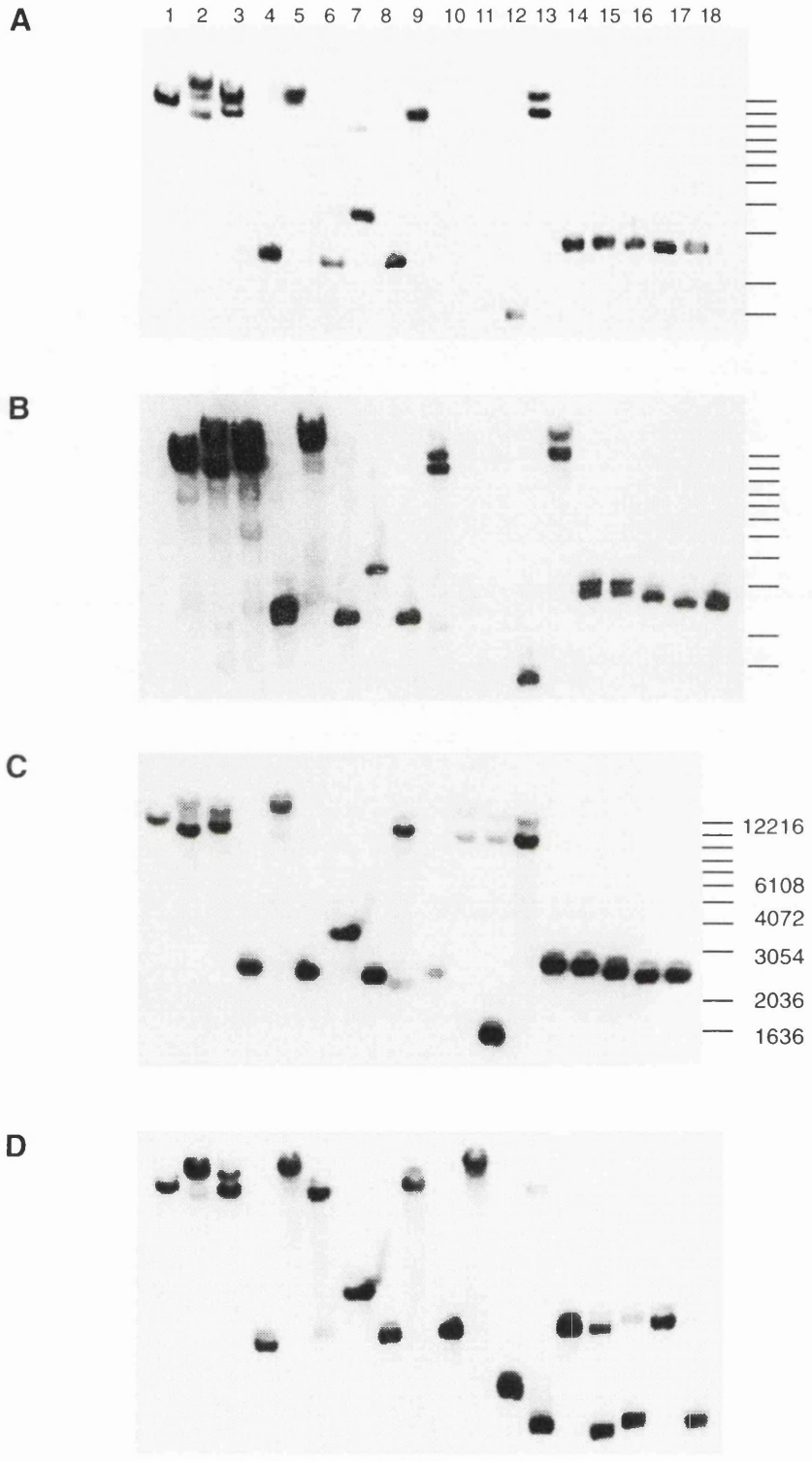
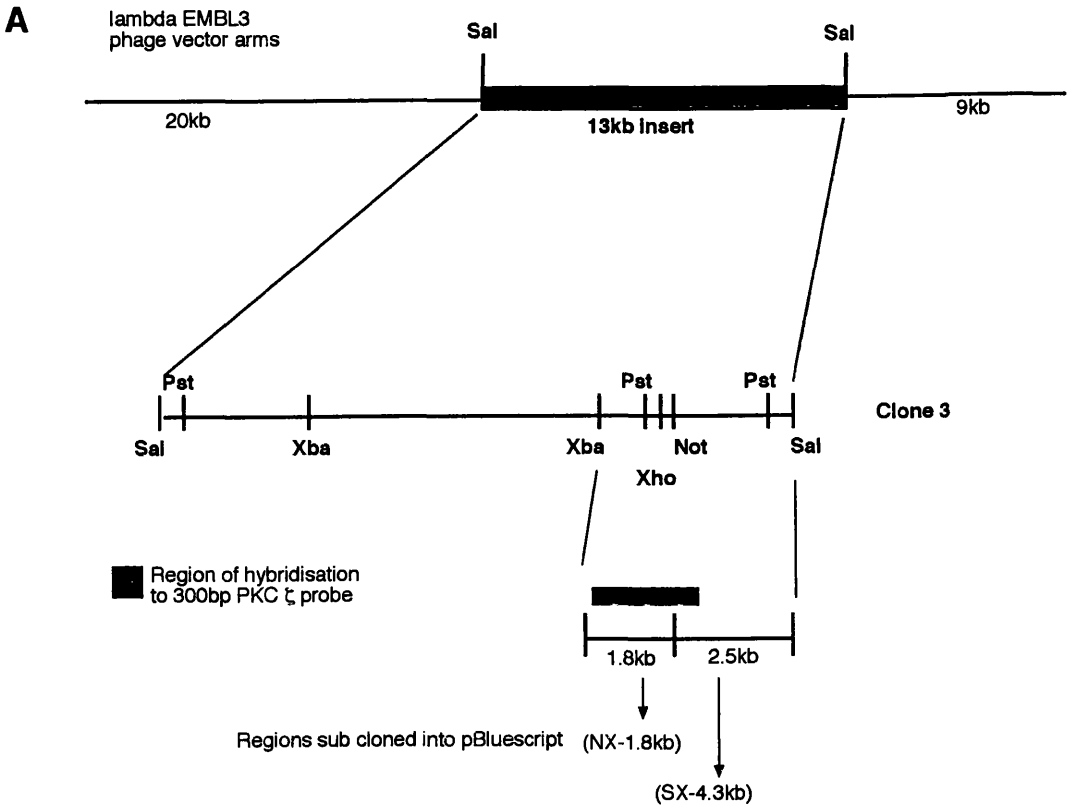


Fig 3.2
Southern blots
All three clones were digested with various enzymes. Southern blots were screened with a labelled cDNA probe to full length PKC ζ (marker sizes given in base pairs).
A. Clone 1, B Clone 2, C. Clone 3, D. Clone 3 (probed with a 300bp N-terminal region of PKC ζ)

The lanes were cut with the following enzymes:- 1.Sal, 2.Xho, 3. Xba, 4. Pst, 5. Not, 6. Sal/Xho, 7.Sal/Xho, 8. Sal/Pst, 9.Sal/Not, 10. Pst/Not, 11. Xho/Not, 12. Xba/Not, 13. Xho/Xba, 14. Xho/Pst, 15. Xba/Pst, 16. Sal/Xho/Xba, 17. Sal/Pst//Xho, 18. Sal/Pst/Xba



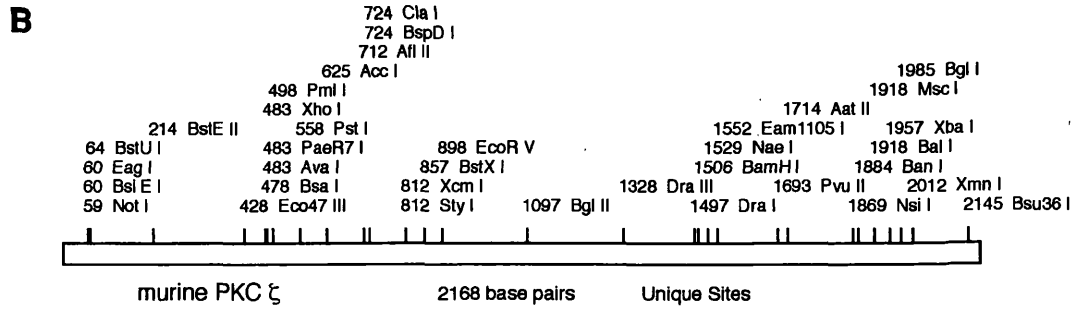


Fig 3.3
Restriction Map of Clone 3
From the size of the restriction products and analysis of double digests, the following map was constructed (Fig 3.3A). The restriction map of PKC ζ is shown below (Fig 3.3B).

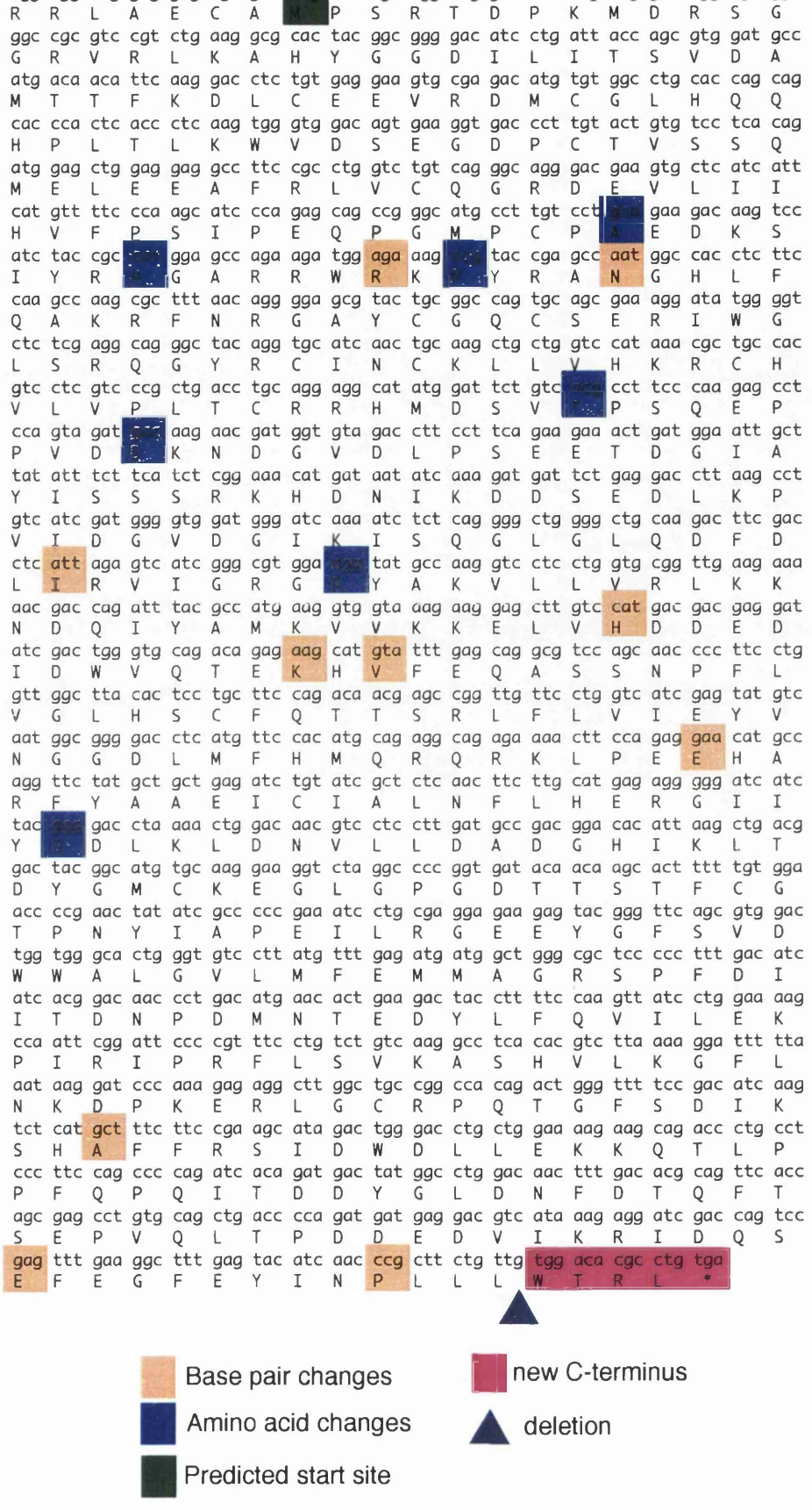


Fig 3.4
Sequence of Clone 3
The sequence was nearly identical to that of PKC ζ and the differences in the ORF region are highlighted.

Table 3.1
A summary of the Differences between Clone 3 and murine PKC ζ

Properties	*Genomic Clone 3*
ORF	1.8kb ORF nearly identical to known murine cDNA intronless gene
mutations	17bp changes
	7 changes at the amino acid level
deletion	40bp C-terminal deletion (switching ORF to give a new 4 amino acid C-terminus)

3.4 Pseudogenes

Pseudogenes closely resemble known genes but tend to be mutated to render them transcriptionally or translationally silent. They usually have multiple mutations, deletions, frameshifts or premature stop codons in their sequence. Processed pseudogenes (or retroposons) are sequences which are reverse transcribed copies of processed mRNAs which have become integrated into the genome (possibly in a mechanism similar to that of LINE transposons (Tchenio et al., 1993). The basic characteristics of a processed pseudogene are:- they are intronless (they appear like mature mRNA); they represent a full length copy of the processed transcript from the functional gene; they contain a 3' poly(A) tail and are flanked by target site duplications (direct repeats) at both ends. Pseudogenes exist in many gene families. The role of these inactive evolutionary "dead end" genes is unknown. However, in human immune responses, pseudogenes play a role in increasing genetic diversity, whereby pseudogene segments can be donated to functional genes, possibly by a somatic gene conversion mechanism (Vargas et al., 1998). Processed pseudogenes are usually inactive (even if they contain an intact ORF) due to lacking active promoters (Adra et al., 1988). However, active processed pseudogenes (or functional retroposons) have been isolated in human and mouse genes, for example, phosphoglycerate kinase 2 (PGK2 (Boer et al., 1987; Gebara and McCarrey, 1992). Genes also exist which are intronless, for example the SRY protein is encoded by a single exon (Behlke et al., 1993). Functional retroposons for another member of the AGC kinase superfamily, PKA, has been isolated (Reinton et al., 1998). PKA has two subunits, catalytic and regulatory, which are encoded by separate genes and further, each subunit has (α–γ) isoforms. The genes are located on separate chromosomes. PKA-C γ subunit is uniquely expressed in the testis and the gene encoding this protein was isolated at the genomic level. Human

genomic PKA-C γ is an intronless gene which is colinear with Cα mRNA and has remnants of a poly(A) tail and is flanked by direct repeats. The role of pseudogenes is unclear. Some pseudogenes are translated. One suggested role for the existence of functional retroposons is that if a gene is expressed specifically in the testis, to overcome transcriptional inactivation during spermatogenesis, a second copy of the gene is found on an autosome. This has been described for testis specific human phosphoglycerate kinase (McCarrey and Thomas, 1987).

To determine if the PKC ζ genomic clone 3 is a pseudogene, two other observations were useful. Firstly, another genomic PKC ζ clone had been isolated (on screening with a full length cDNA probe) and this gene contained introns (personal communication from Silvia Stabel in Cologne). Secondly, a report was published describing another PKC ζ pseudogene, ΨPKCζ (Andrea and Walsh, 1995). This pseudogene is not the same as the one isolated here. ΨPKC ζ was isolated after screening a rat brain cDNA expression library using a polyclonal antibody to the C-terminal region of PKC ζ (GFEYINPLLLSAEESV). The pseudogene was 2661 bp long and contained a different V1 region to PKC ζ, V1', which is identical to the original short sequence isolated by Ono and colleagues (Ono et al., 1988). ΨPKC ζ is transcribed but is reported not to be translated since it does not have a methionine start codon at the beginning of the sequence. Together, these observations suggested that PKC ζ pseudogenes do exist and the authentic gene contains introns.

If Clone 3 is a pseudogene, denoted PKC ζ Ψ I here, which is suggested by the 3' poly(A) tail (a sign that the processed mRNA of PKC ζ became integrated into the genome), then the age of divergence and transposition can be calculated. Based on the "neutral theory of evolution" (Kimura, 1983), the evolutionary time that has elapsed since the divergence of a pseudogene from the original gene can be estimated. The assumption is that after silencing, the rate of mutation of the pseudogene is close to that of spontaneous mutation, which is 3.7×10^{-9} substitutions per year. Of course this is assuming that the mutations did not arise before the gene became inactivated and is not true if this turns out to be a new gene as opposed to a pseudogene. If the deletion in the C-terminus is also included, there are 34 bp changes in this pseudogene in comparison to PKC ζ and the divergence occurred relatively recently, 5.2 Myr ago. This potential PKC ζ pseudogene formed very recently.

3.5 Characterisation of the Pseudogene

3.5.1 Chromosomal Localisation

What is the origin of the genomic clone? Was the gene formed by alternative splicing? Does Clone 3 encode a distinct gene from the authentic gene? These questions were partially answered by analysing the chromosomal localisation of the two genes. FISH (Fluorescence *In Situ* Hybridisation) was used to map the location of probes from the pseudogene and the authentic gene (given by S.Stabel) on mouse metaphase spreads. The different probes used are outlined in Figure 3.5. The FISH analysis (Figure 3.6 and Figure 3.7) was carried out by Jill Williamson in the ICRF Human Cytogenetics Laboratory (Monier et al., 1996; Ragoussis et al., 1992).

The ZETA and pz-X5 probe hybridised strongly to one locus at the proximal end of one chromosome and weakly to a central region of another (Figure 3.6). The SX1 probe was too small to hybridise efficiently, however the 3' untranslated region of the pseudogene was sufficient to determine the locus of the pseudogene. The intron sequence of PKC ζ (pz-X5 probe) should hybridise solely to PKC ζ (not PKC λ) and it is interesting to see two hybridisations. The second binding was weaker but reproducible.

Chromosomal paints were used to identify the chromosomes which the probes were bound to (Figure 3.7).

Table 3.2 outlines the chromosomal localisations of PKC ζ. The authentic gene is on Chromosome 4 (and faintly on the X chromosome) and the pseudogene, PKC ζ Ψ I, is localised on Chromosome 7. Clearly the pseudogene has a distinct chromosomal location. This rules out the possibility that the "pseudogene" isolated formed by differential splicing from the authentic gene or some aberrant library construction. The new C-terminus may have formed by alternative splicing of the original gene before retroposition occurred. How the gene evolved cannot be established.

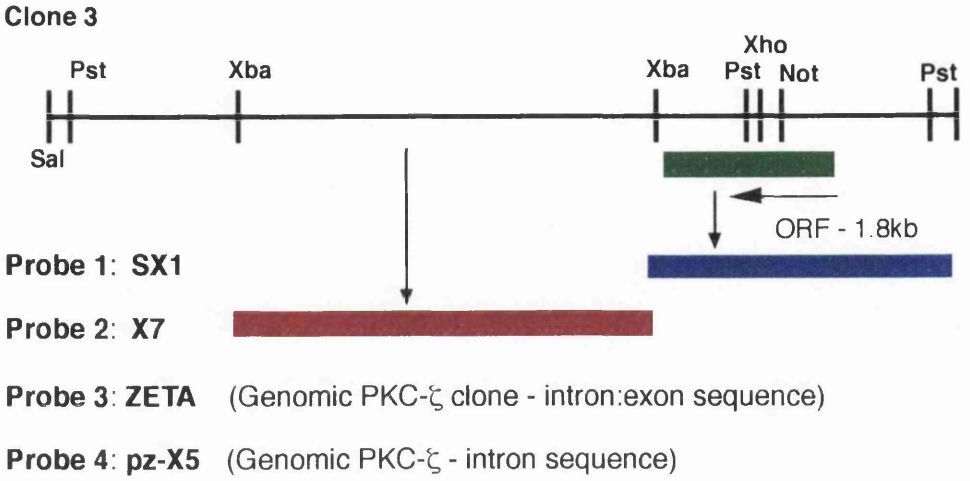


Fig 3.5
Probes used for FISH
Various DNA regions were used as hybridisation probes. The pseudogene was identified by SX (ORF) and X7 (5' untranslated region). Two probes were used to recognise the authentic gene - ZETA and pz-X5.

Fig 3.6

Chromosomal localisation of the Pseudogene and PKC ζ

Probes hybridised to specific loci on different chromosomes (green spots) and are indicated by arrows. For the ZETA probe, hybridisation occurred on two different chromosomes, one hybridisation region in the middle of a chromosome, the other at the proximal end (Fig 3.6A). Similar hybridisation patterns were seen for pz-X5 (Fig 3.6B).

Fig 3.7

Chromosomal Paints identify the Chromosomes which PKC ζ probes hybridise to

The regions that the various PKC ζ probes hybridised to clearly matched the specific chromosomal paints. Hybridisation regions are shown on the left hand side (as green spots). These correspond to the same metaphase spreads incubated with specific chromosomal paints, shown on the right.

A. X7 probe and chromosome 7 paint
B. pz-X5 probe and chromosome 4 paint
C. ZETA probe and X chromosome paint

A

B

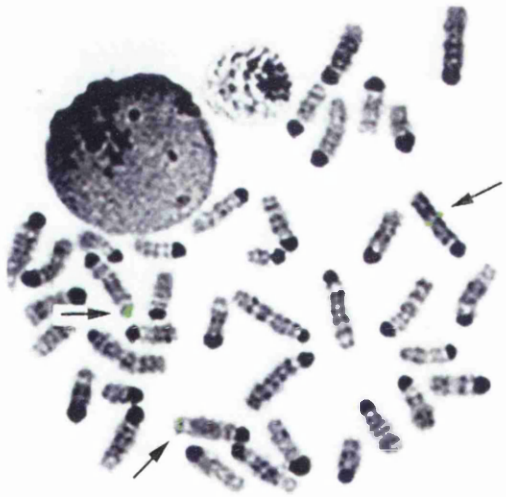


Fig 3.6

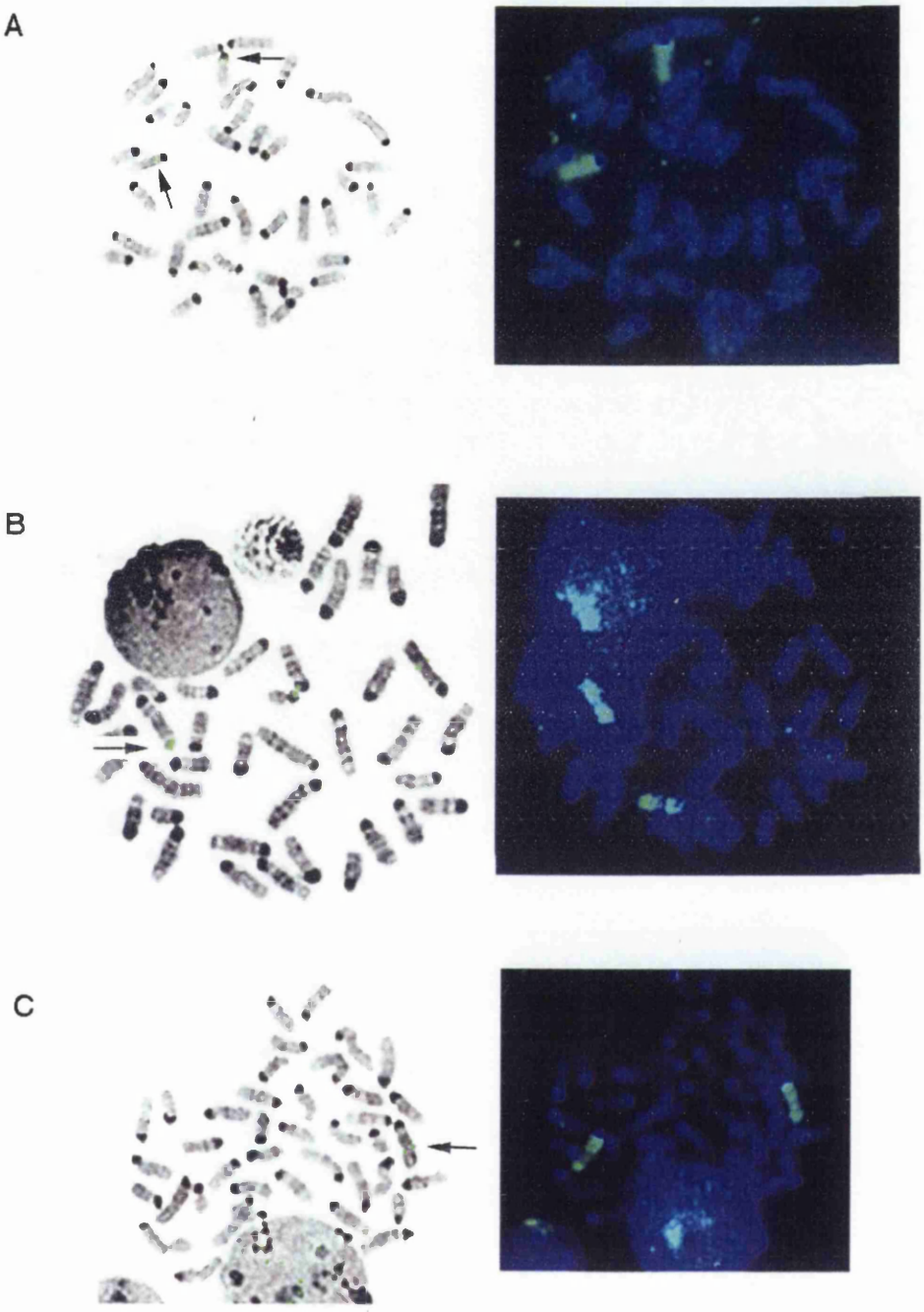


Fig 3.7

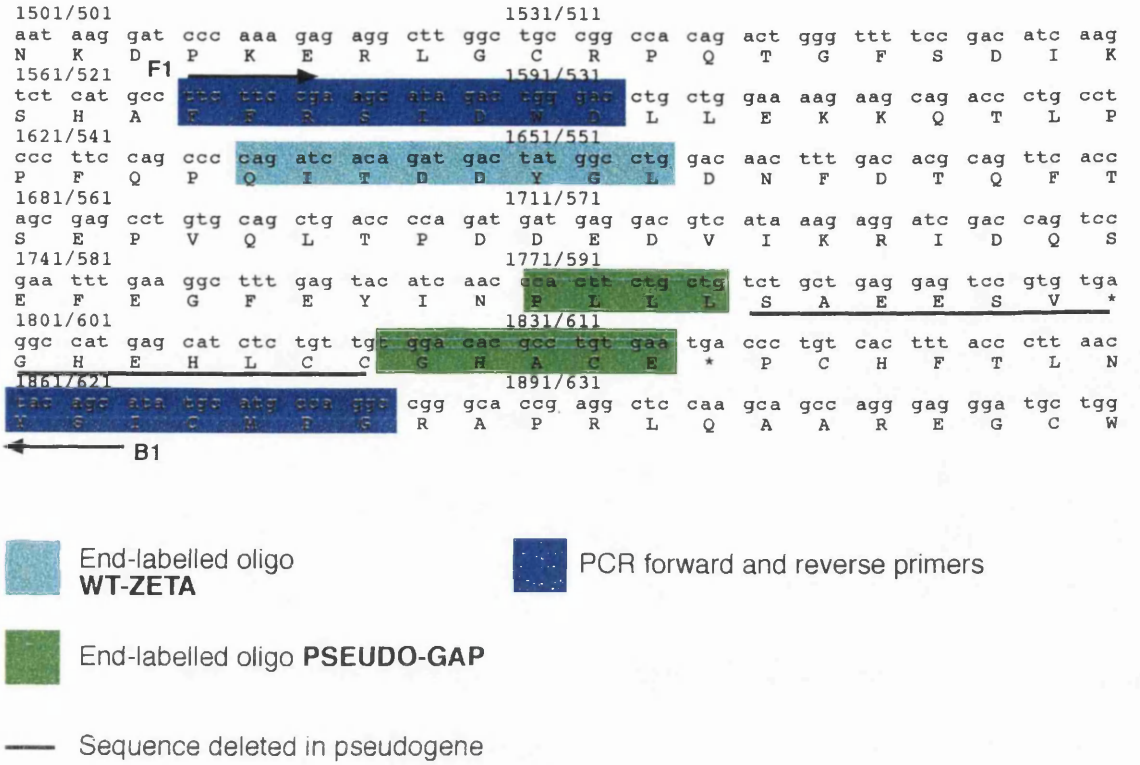
Table 3.2
Chromosomal Localisation of PKC ζ and PKC ζ Ψ I

Probe	*Classification*	*Chromosomal localisation*
pz-X5	intron/exon seq.	Chr 4 (E2 region)/ X Chr (centre)
ZETA	intron seq.	Chr 4 (E2 region)/ X Chr
X7	pseudogene	Chr 7 (A2-3 region)

3.5.2 Transcription

The next issue to be tackled was:- is the pseudogene transcribed? The problem with trying to separate the pseudogene from the authentic gene transcripts was that the only distinguishing feature between the pseudogene and the authentic gene is the C-terminal deletion. Therefore, PCR was carried out on six cDNA libraries (kindly received from Dr D. Simmons' laboratory, ICRF) to determine if PKC ζ Ψ I or a truncated or spliced PKC ζ gene can be transcribed. Primers were designed around the deleted region (Figure 3.8).

The products were analysed on 4% gels and then transferred for Southern blot analysis. The DNA gels clearly show two bands being produced (Figure 3.9A) suggesting that the smaller band (265bp) is the pseudogene and the larger (305bp) PKC ζ. Southern blots were probed with various end-labelled oligos to attempt to distinguish between the two bands. The results are rather inconclusive because all the end-labelled oligos hybridised to give broad bands (on autoradiography) around the 298 marker and it was difficult to distinguish between the two possible fragments. The different oligos showed similar hybridisation patterns (Figure 3.9B,C). A sequence resembling the pseudogene certainly appears to be transcribed and was expressed in two tissues analysed (thymocyte, splenocyte and very faintly in Swiss 3T3 fibroblasts).



End-labelled oligo **WT-ZETA**

PCR forward and reverse primers

End-labelled oligo **PSEUDO-GAP**

Sequence deleted in pseudogene

Fig 3.8
PCR primers and oligos used to analyse the cDNA library transcripts

PCR was carried out over the C-terminal region of PKC ζ to try to isolate two transcripts (the authentic gene and pseudogene). PCR primers are shown and oligos used for Southern blot probing are highlighted.

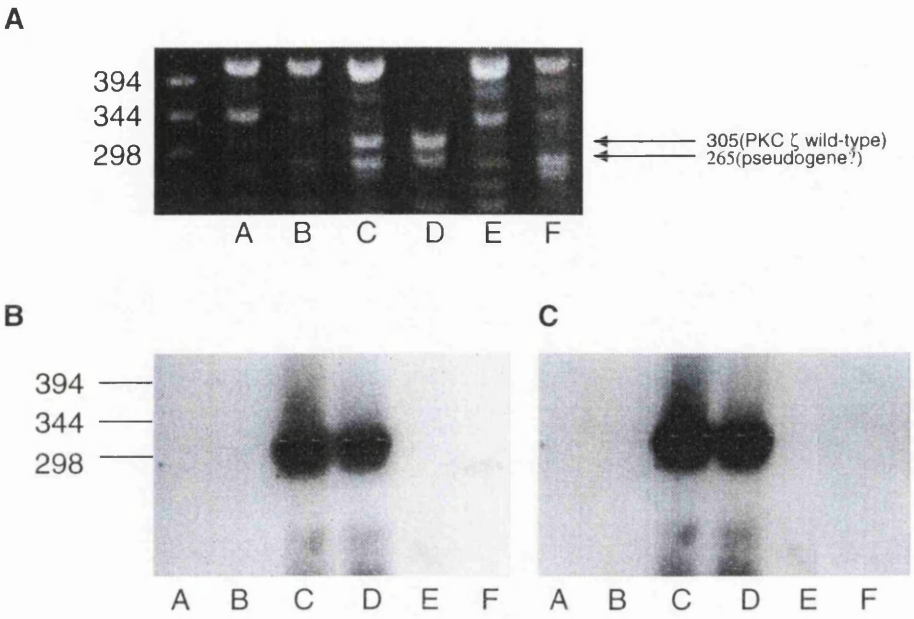


Fig 3.9
PCR products - end labelling
PCR was carried out over the C-terminal region. The DNA gel (Fig 3.9A) was transferred and the Southern blot hybridised with two end-labelled oligos - PSEUDO-GAP (Fig 3.9B) and WT-ZETA (Fig 3.9C). The size of the two transcripts is indicated.
cDNA libraries - lanes:- A. bone marrow, B. endothelium, C. thymocyte, D. splenocyte, E. melanoma, F. Swiss 3T3

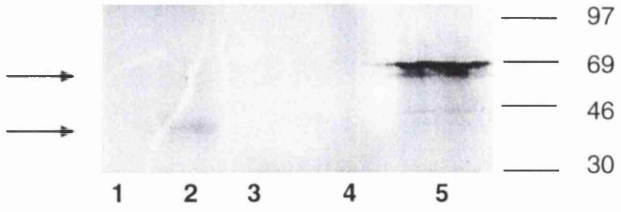


Fig 3.10
***In vitro* transcription-translation**
Various pseudogene constructs were expressed in a rat reticulo lysate *in vitro* transcription/translation system. A protein of size around 45kD and a very faint protein of 65kD was identified.
Constructs expressed, lanes:- 1. NX (Not/Xba - no ATG), 2. SX Δ (deleted 5' untranslated region) 3. SX full (full length clone), 4. X7 (3' untranslated region), 5. luciferase control.

3.5.3 Translation

If there are pseudogene transcripts and there is an intact ORF, there is potential for the protein to be produced. The following experiments were carried out to see if a protein from the ORF is translated and is stable. *In vitro* transcription-translation suggested that a protein could be produced (Figure 3.10). Only on removal of the 5' untranslated region is the ORF translated. The extended time of the incubations may explain why the more stable but smaller kinase domain is seen and the full length protein is only faintly visible. PKC ζ itself is very sensitive to proteolysis and is easily degraded to give a 47kD catalytic fragment (Ways et al., 1992). The next step taken was to determine if the ORF can be expressed in COS cells. Several constructs were transfected into COS cells (in SXΔ, a 5' untranslated 2.5kb fragment was removed before cloning).

Initially, a commercial antibody, which recognises a slightly larger epitope (Figure 3.11), was used to see if the ORF of the pseudogene (which had been cloned into a mammalian expression vector - pcDNA3) could be detected in cells. The ICRF PKC ζ antibody will not detect the pseudogene as strongly as the wild-type protein. A protein of approximately the right size, around 75kD, was detected strongly with the commercial antibody (and seen by coomassie staining) but poorly by the ICRF PKC ζ antibody (Figure 3.12).

Clearly, this gives no indication of whether the pseudogene has an operational promoter *in vivo*. To address this issue, fractions from a rat brain preparation were screened with a pseudogene-specific antibody.

Most PKCs are abundantly expressed in the brain and the most mRNAs for PKC ζ are found in the brain. The other pseudogene was also isolated from the brain. Therefore, rat brain fractions were screened. The rat brains were homogenised and then centrifuged to give a pelleted membrane fraction (membrane) and supernatant (cyt-Hep). The membrane fraction was then passed over a Mono Q column and any material that did not bind was collected in the salt wash. The supernatant was passed down a heparin column and protein eluted with 0.5 and 2.0M NaCl.

The commercial antibody detects two bands (Figure 3.13A). By using the ICRF PKC ζ antibody, one is clearly PKC ζ and the other is possibly the pseudogene. This was confirmed using the pseudogene antibody (Figure

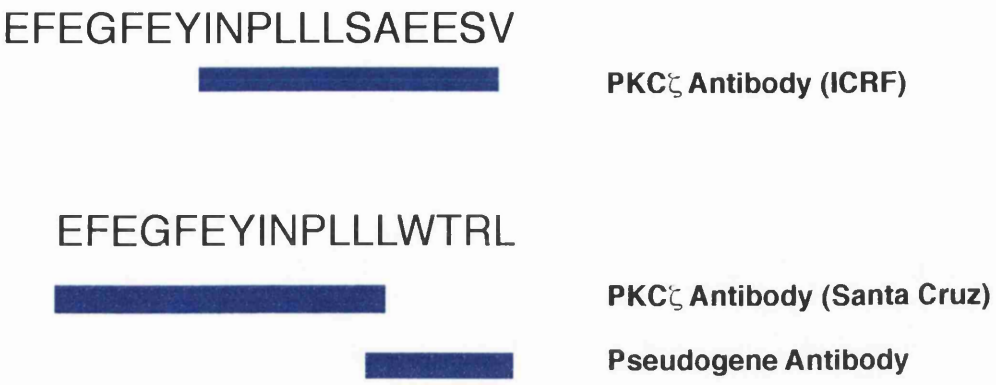


Fig 3.11
Antibodies
Three antibodies were used to distinguish the various PKC ζ protein products. The PKC ζ antibody produced in the lab only recognises the carboxy terminus of PKC ζ.

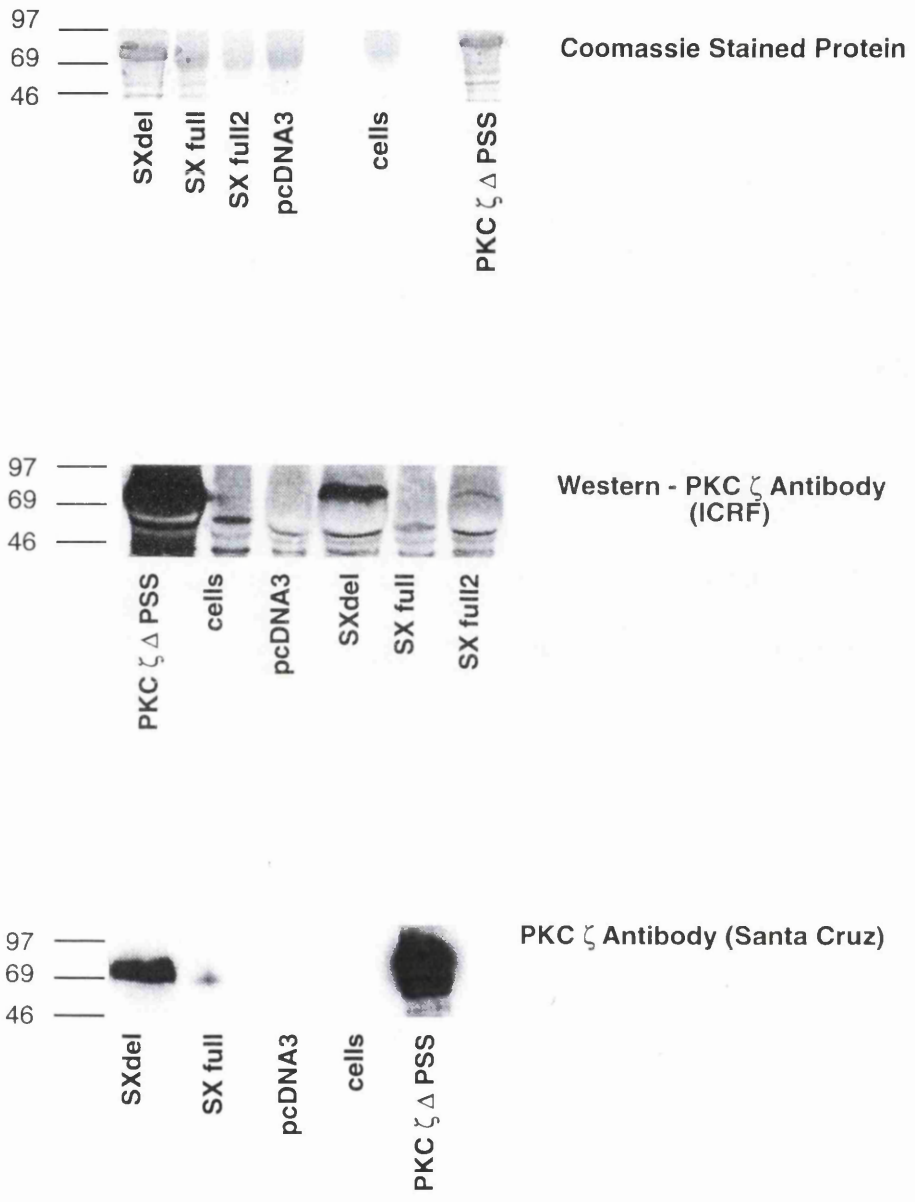


Fig 3.12
Expression of a Pseudogene Protein
COS cells were transfected with various pseudogene constructs and constitutively active PKC ζ as a control. The gel was coomassie stained or blotted and incubated with both PKC ζ antibodies. A protein of around 75kD was identified.

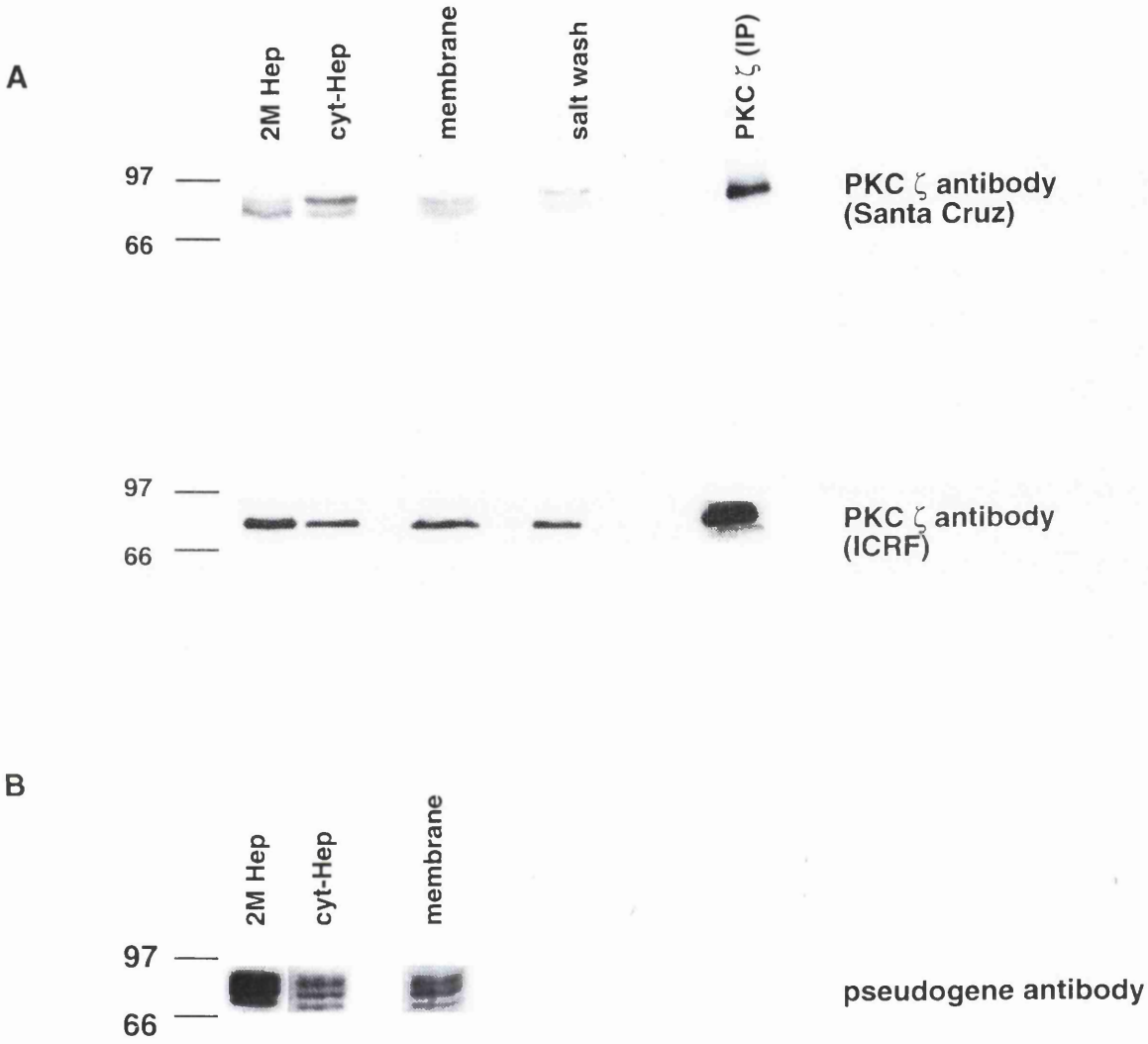


Fig 3.13
Pseudogene - a protein?
Rat brain fractions were blotted with both PKC ζ antibodies (Fig 3.13A) and the pseudogene antibody (Fig 3.13B). Fractions were obtained from a large scale rat brain protein extraction.

3.13B). Several bands were isolated (it is not known what else the antibody can recognise) which were also competed away with peptide. Analysis of the LWTRL peptide against Swissprot database did not align with any C-terminal sequences and the only proteins containing this sequence were of bacterial or yeast origin. This suggests that the pseudogene is transcribed and a protein is produced.

3.6 Discussion

A genomic clone was identified using a PKC ζ probe. The work described in this chapter establishes that this may be a novel PKC ζ pseudogene, PKC ζ Ψ I. From the chromosomal localisation data, the pseudogene and the authentic gene are on two different loci. The different chromosomal localisations show that the pseudogene is real and is not a cloning artefact. The different loci suggests transposition of the gene. Other PKC genes have been localised to specific chromosomes (Table 3.3). Interestingly, other murine PKCs have been localised to chromosome 7. PKC γ is potentially in the same region as the pseudogene. However, what lies upstream of the pseudogene in the remaining 5' untranslated region is unknown. How the location relates to its evolution or role of the gene is also not known. Often certain genes may form clusters together. Certain chromosomal loci have been linked to various diseases. For example, the locus of PKC ι has been closely linked to the Btk gene and defects in this latter gene results in an immunodeficient syndrome, agammaglobulinemia. Even though the two genes have not been shown to be related, their proximity on the X chromosome is intriguing. If the murine PKC ζ loci are assigned approximate human loci, no known diseases have yet been associated with these positions.

Table 3.3
Chromosomal Localisations of Atypical and other PKCs

PKC	*murine gene*	*Chromosomal localisation*			*References*
		Human	*Mouse*		
PKC α	Pkca	17 q22-q24	11	68	(Summar et al., 1989) (Tang and Ashendel, 1990)
PKC β	Pkcb	16 q11.2	7	60	(Francke et al., 1989) (Coussens et al., 1986)
PKC γ	Pkcc	19 q13.2-13.4	7	2	(Saunders and Seldin, 1990) (Johnson et al., 1988)
PKC δ	Pkcd	3 pter- qter	14	15.5	(Huppi et al., 1994)
PKC η	Pkch	unknown	12	C3-D1	(Canzian et al., 1994)
	Pkch-rs1/2		12	29, 31	(Osada et al., 1990)
PKC θ	Pkcq	10 p15	2	2	
PKC ι		X q21.3			(Mazzarella et al., 1995)
PKC λ	Pkcl		3	D3	(Quaderi et al., 1995)
PKC ζ		unknown	4	E2	
ΨPKCζ			X		prediction
PKC ζ Ψ I		unknown	7	A2-3	this chapter
PRK1		16 p11-12			Palmer

The evidence suggests that PKC ζ Ψ I has characteristics of a processed pseudogene. There are no introns (it is known that PKC ζ has introns at the genomic level, observations from S.Stabel's group); there are various mutations in the whole sequence and a deleted C-terminus, and there is a poly(A) tail in the 5'untranslated region. The intron-exon boundaries of aPKCs have not yet been clarified. It would be interesting to discover if there was an intron near the end of the ORF, which may explain the origin of the new C-terminus. Recently, a novel cDNA in *C.elegans* was cloned which encodes an aPKC gene, PKC3 (Wu et al., 1998). PKC3 appears to be the only aPKC in *C.elegans* and is a composite protein of PKC ζ and PKC ι (overall 55% and 57% homology, respectively). However, the intron-exon boundaries could be analysed from the *C.elegans* genome database. PKC3 consists of nine exons separated by introns of varying sizes. The final exon is

from amino acids 557-597, which excludes the possibility of a splice site at the point of the deletion. The introns may be larger in mammalian homologues, being a minimum size of 66-70 bp in size (Smith et al., 1989). How strongly intron-exon boundaries are conserved between species is unknown. However, the intron-exon structure of the human PKC β (PRKCB) gene has recently been analysed (Greenham et al., 1998) and compared to *Drosophila* PKC (Rosenthal et al., 1987). The PRKCB ORF consists of 18 exons, including the two alternatively spliced C-terminal regions of PKC βI and PKC βII and encodes a region of 375kb. In comparison, dPKC (with 64%identity to PKC α) comprises 13 exons over a region of 20kb. The splice sites are conserved between species, however, dPKC exon 7 is divided into 3 exons in PRKCB and dPKC exons 10 and 11 are subdivided into 4 PRKCB exons. One of the dPKC exons contains 5' untranslated sequence. Interestingly, even though the ORF sequence are similar in size (2172 bp for PRKCB and 1920bp for dPKC) the average intron size is much larger for the human gene (22kb in comparison to 1.5kb in *Drosophila*). The difference in gene size can be correlated to the difference in genome size, where the haploid genome of *Drosophila melanogaster* (170Mb) is 18 times smaller than the human haploid genome (3000Mb). Furthermore, C.elegans PKC3 intron-exon structure is very similar to that of dPKC. PKC3 has 9 exons, lacking a few which encode the second zinc finger and the C2 domain. Thus, the intron-exon structure for the PKCs appears to be essentially conserved.

One transcribed PKC ζ pseudogene (ΨPKCζ) has already been described. This was identified from a rat brain screen (Andrea and Walsh, 1995) and was also isolated by another group (Powell, 1994) from rat prostatic tumours. Interestingly, this pseudogene was the first identified form of PKC ζ (Ono et al., 1988). When rat PKC ζ was originally isolated, a clone without a start codon and having a 28bp non-PKC N-terminus was isolated. Therefore they hypothesised that this non-PKC N-terminus arose from lack of splicing of an N-terminal intron, however they did not show any data to prove this idea. The two groups who subsequently isolated the pseudogene carried out RNAse protection assays and genomic PCR over this V1'-C1 region (V1' is the novel 214bp N-terminal non-PKC sequence). This region is a continuous and uninterrupted region of the genome. Distinct hybridisation patterns from genomic Southerns (using pseudogene and PKC ζ specific probes) indicate the pseudogene and PKC ζ are distinct genes. Moreover, when the human and murine PKC ζ cDNAs were isolated, again both full length clones were not isolated at the first attempt (Goodnight et al., 1992). How these pseudogenes formed and what the chromosomal locus of the ΨPKCζ gene

is, will be interesting to identify. It is possible that the faint hybridisation on the X chromosome seen with the PKC ζ genomic probe is this pseudogene, ΨPKCζ. This could be readily clarified if the location of the sequence of the PKC ζ genomic probe within the PKC ζ ORF is known.

The data from Northern blot analysis has not been very straightforward. Walsh *et al*, who isolated the ΨPKC ζ, found that the mRNA which has always been associated with PKC ζ, 2.2 and 4.2kb, was actually associated with the pseudogene (Figure 3.14). Between species and in other non-brain tissues, the mRNAs differ in size by a few hundred bases. However, the group also isolated an mRNA of 1.75kb with a probe to the C1 region. Saktor *et al* isolated a truncated PKC ζ mRNA specifically in the brain (ASBMB meeting abstract, 1998). It will be interesting to determine if these two transcripts are the same, encoding PKMζ. The discovery of several different mRNA transcripts implies that PKC ζ uses either different polyadenylation sites and different splice variant forms, as is seen for PKC βI and PKC βII, or that different sub-isoforms (i.e. genes) exist. For the classical PKCs, several mRNA transcripts are formed and for PKC β and PKC γ, less transcripts arise in the brain. Potentially several alternative splice variants, pseudogene or novel genes may exist for all other PKCs.

If both the PKC ζ pseudogenes are indeed transcribed and translated, it will be intriguing to understand function. The ΨPKCζ is transcribed, but there is no obvious methionine at the beginning of the sequence. However, there are two stop codons 10-20bp upstream of the SIY PKC ζ start sequence. A subsequent ATG codon at amino acid 184 could possibly become a start codon encoding a catalytic domain protein, PKMζ. Sacktor originally proposed that a PKMζ 51kD protein is produced during LTP (Sacktor et al., 1993). It was unknown whether PKMζ is expressed as the catalytic domain alone or is just formed by protease cleavage of PKC ζ under increased calcium levels, caused by LTP. Recently the same group have used antibodies to various regions of PKC ζ and have demonstrated that PKMζ is more abundantly expressed in the brain than PKC ζ. Thus the ΨPKCζ pseudogene may be translated into a PKM form and be a novel PKC ζ isotype.

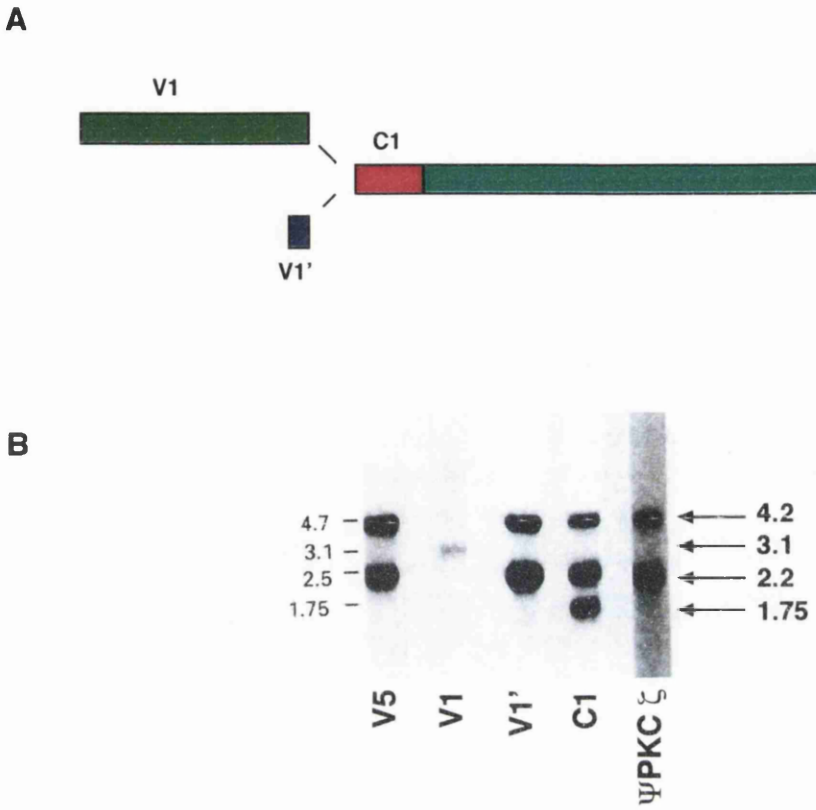


Fig 3.14
Northern blot analysis of mRNA from rat brain
Specific regions of PKC ζ were used to hybridise rat brain mRNA. The probes were specific to PKC ζ (V1, C1 or V5) or the pseudogene (V1') and the full length pseudogene cDNA (ΨPKC ζ) Fig 3.14A.
Results taken from (*Biochem.J. (1995) 310 835-843 J.E.Andrea,M.P.Walsh*) Fig 3.14B.

In the case presented here, it would appear that PKC ζ Ψ I may be translated too so it may also be a novel PKC ζ isotype. Only future experiments will be able to define if the transcript is translated. The following would help clarify transcription and translation.

- extend the PCR analysis to identify which tissues express the pseudogene transcript (using the C-terminal PCR approach explained here and identifying the pseudogene by sequencing)
- study the endogenous pseudogene by using the antibody to detect or immunoprecipitate protein in any tissues known to express the transcript
- further characterise the pseudogene protein in 293 cells by immunoprecipitating the pseudogene protein and testing for activity and by immunofluorescence, to see if the new C-terminus changes the cellular localisation of the protein
- discover the origin of the pseudogene, by sequencing more bases of the untranslated regions looking for 5' and 3' splice site/direct repeats for retroposition
- isolate (either by placing the 5' untranslated region in front of a Lac Z etc gene or sequence analysis) a promoter
- study the pseudogene expression in the PKC ζ knockout mouse tissues where there is no authentic PKC ζ expressed

If the pseudogene (PKC ζ Ψ I) or any other PKC ζ variants are expressed, this will affect the outcome of any PKC ζ knockout mice. Libraries were not re-screened to pull out an authentic PKC ζ gene because Sylvia Stabel's group were already injecting a PKC ζ construct into embryonic stem cells. To avoid duplicating research, a PKC ζ knockout was not pursued. M. Leitges from this group has now characterised several PKC knockouts (Table 3.4).

Table 3.4
PKC knockouts (homozygote -/- mice, unpublished data from M.Leitges)

PKC isotype	*Phenotype*
PKC α	normal - possible effect on platelets
PKC β	depleted B cell populations/ learning or behavioural defect? (Leitges et al., 1996)
PKC γ	Effects on LTP (Abeliovich et al., 1993; Abeliovich et al., 1993)
PKC δ	effects on brain? / wound healing defects/ die in 6 months
PKC ε	no overt phenotype (data from Dr M. Owen, ICRF)
PKC η	defects in wound healing
PKC ζ	no overt phenotype
PKC ι	embryonic lethal (day 7)
PKD (PKC μ)	effects on T cells

Knockouts can be informative in elucidating the biological role of a protein. The PKC knockouts themselves have not given much insight into the role of the specific PKCs since the defects are more subtle. Differences may only be noticeable at the cellular level or under certain conditions, for example, PKC δ may be involved in recognition of invading pathogens. The isoform which causes the most striking phenotype is PKC ι. Embryonic lethality may imply that the protein is required for embryonic development or that it plays a vital role in the functioning of cells. PKC3, which is a composite aPKC in *C.elegans,* is essential for the progression of embryogenesis. If functionality is conserved across species barriers, it suggests that PKC ι plays an important role in embryogenesis. The other problem is that in such a family as the PKCs, there may be redundancy and double knockouts may give more clear-cut answers. The potential to study different cell types deficient in specific PKCs and the effects on cellular function is immense. In the future, making more subtle knockouts, conditionals (Lobe and Nagy, 1998) or fusion with GFP/lac Z or other specific targeting proteins, to visualise, localise and specifically switch on the knockout, will help overcome the problem of embryonic lethality, as seen for PKC ι.

Transgenic mice will help solve the question of the biological role of proteins in the context of an organism. It would be interesting to set up transgenics using constructs of the activated (or kinase dead) PKCs, for example, either being conformationally open by deleting the pseudosubstrate region, or by

using point mutations of the phosphorylation sites which appear to make PKC ζ active *in vivo* but less stable *in vitro* (see the following chapter).

However, in the future, especially in trying to decipher the PKC ζ knockout phenotype, it will be important to discover if functional PKC ζ is encoded by a subgroup of genes. Potentially there are three PKC ζ genes :- PKMζ or ΨPKC ζ; PKC ζ and the PKC ζ Ψ I, described in this chapter. The atypical PKC family may now be expanding to encompass two new kinases.

Chapter 4

4.1 Introduction

With the knockout of PKC ζ awaiting progress from others, the role of PKC ζ was pursued by elucidating its control and so helping to isolate at least one signalling pathway on which it lies. How aPKCs are regulated and how they fit into cellular signalling pathways will be investigated in the following chapters. The approach to this problem was derived from the structural similarity between PKC ζ and other PKCs, in particular, focusing on conserved phosphorylation sites.

Allosteric effectors and phosphorylation is required to activate PKCs (see Introduction). For the cPKCs, three "priming" sites have been mapped. The activation loop site is essential for maximal catalytic competency. In addition to the permissive phosphorylation of this site, the two other priming sites required for PKC α activity are the T657 site, in a hydrophobic region, and T638 (S660 and T641 respectively for PKC βII). How these phosphorylations are regulated is controversial. Papers published by A. Newton's group give evidence for the S660 site in PKC βII being an autophosphorylation site (Keranen et al., 1995). However, work from our lab demonstrates that this site is trans-phosphorylated in PKC α (and from work on PKC δ) by an as yet unidentified kinase. This site is highly conserved in the AGC kinase superfamily and certainly for PKB (and $p70^{S6K}$ where phosphorylation of this site is sensitive to rapamycin), a distinct kinase is believed to phosphorylate this site. It could be hypothesised that there is a common kinase for this site. Recently integrin linked kinase, ILK, was identified as a candidate for phosphorylating S473 in PKB (Delcommenne et al., 1998).

The role of these C-terminal phosphorylation sites was investigated by Frederic Bornancin by making PKC α phosphorylation site mutants. Mutation of the T638 and S657 sites does not directly affect catalytic activity. Phosphate incorporated into these two sites has a more subtle role. The T638A and E mutants were more sensitive to phosphatases (PP1 *in vitro*); less thermally stable and more open to oxidation in comparison to the wild-type protein (Bornancin and Parker, 1996). This led to the hypothesis that phosphorylation affects the conformational state of the protein (Figure 4.1). If all sites are occupied, the catalytic domain is in a more closed conformation.

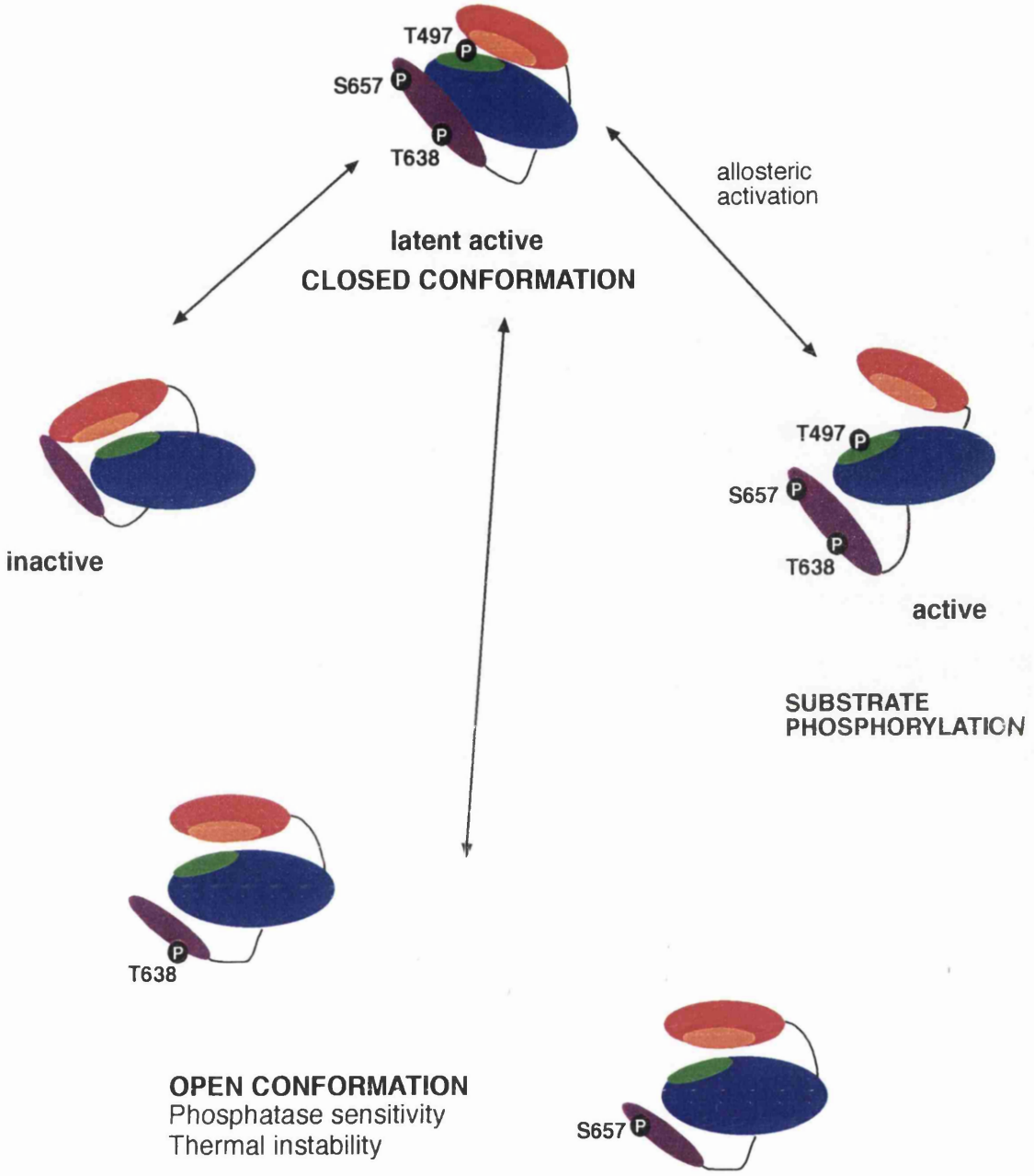


Fig 4.1
Model for the Role of Phosphorylation in determining the conformational state of PKC α

This reflected what was seen on TPA treatment *in vivo*. TPA stimulated mutants were more prone to dephosphorylation by phosphatases than the wild-type protein. The increased exposure of certain sites may also explain the increased sensitivity of the mutants to oxidation. A comparison to PKA helps understand this problem. In PKA, a cysteine residue in the activation loop interacts with the γ-phosphate of ATP in the active site. If this Cys (C199) is covalently modified, activity is lost. For PKC α, the equivalent site may be C499. C499 may be more exposed in the phosphorylation site mutants and so can be readily oxidised, resulting in loss of activity. Presumably this residue is buried in the wild-type protein which protects against oxidation. The specific activities of isolated mutants from unstimulated cells are listed below (Table 4.1). Clearly the C-terminal mutants do not affect activity greatly *per se*. As expected, an Ala residue in the T497 site results in loss of activity. Interestingly *in vitro,* isolated protein with threonine mutated to a glutamic acid (T497E) does not result in complete mimicking of a phosphate group.

Table 4.1
Specific Activities of PKC α mutants

Phosphorylation site Mutants	***Activity*** *(with respect to the wild-type protein)*
T497E	22%
T497A	0
T638E	71%
T638A	74%
T657E	80%
T657A	45%

From Western blot analysis and pulse chase labelling experiments, using the S657A or D mutants, it was obvious that phosphorylation in this site controlled and is a rate determining step for phosphorylation of the other sites. The S657A mutant never accumulated in a fully phosphorylated form following ^{35}S-methionine labelling and chase of the proteins. However, the primary translation product of the S657D mutant is initially processed more rapidly (running faster than the wild-type protein, 78kDa in an unphosphorylated state). Therefore, the S657 site controls two separate processes. It appears to be the first (or one of the first) phosphorylation events resulting in the subsequent phosphorylation of all other sites. Secondly, similarly to the T638 site mutants, incomplete phosphorylation renders the

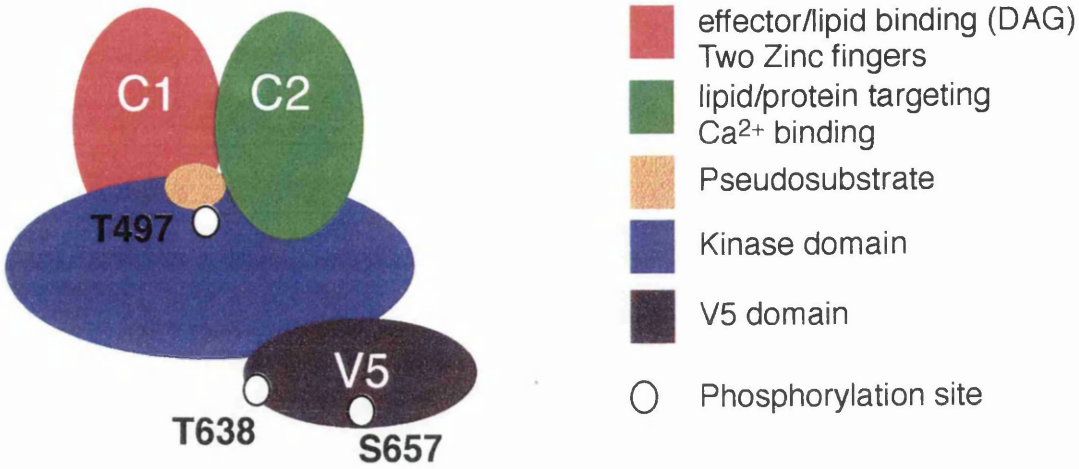
protein more accessible to phosphatases and degradation (Bornancin and Parker, 1997).

Phosphorylation in the two C-terminal sites does influence phosphorylation in the catalytic core and co-operativity between sites is observed. This may be explained by the carboxy terminal region of PKC α "masking" the activation loop and being wrapped across the T497 site. The initial event may require S657 phosphorylation, releasing the V5 C-terminus from the activation loop, thus enabling the second PKC kinase to phosphorylate T497. Subsequently, the T638 site may make contact with a cluster of basic residues in the smaller, upper lobe of the kinase domain, thus stabilising the catalytic domain. This would explain the co-operativity of interaction and phosphorylation. The mechanistic details of the phosphorylation events have not been analysed further.

How does our knowledge of the phosphorylation sites of PKC α and PKC βII relate to other PKCs? Can the same ideas be applied to the novel and atypical PKCs? Experiments for PKC δ demonstrate that the S643 site (equivalent to T638 in PKC α) is an autophosphorylation site (Li et al., 1997). However, S643A protein isolated *in vitro* and tested for activity was not thermally unstable but had reduced enzymatic activity and autophosphorylation. Thus, this may correlate with the idea that lack of phosphorylation in this site affects protein activity indirectly due to incorrect alignment with the activation loop site. TPA-induced monocytic differentiation of 32D cells overexpressing the Ala mutant still occurs but to a lesser extent than for the wild-type protein. Therefore, the mutant is less efficient in acting on key substrates in the differentiation pathway. There is no data on what happens to S662A or E mutants for PKC δ.

For the aPKCs, there is no published data on phosphorylation sites. Comparatively, aPKCs can undergo phosphorylation in two of the three priming sites (Figure 4.2). S657 residue is a glutamic acid in the aPKCs, thus this primary phosphorylation step is bypassed. It was considered important to discover what difference this makes to the regulation of the aPKCs by phosphorylation. How these sites affect atypical PKC protein stability and activity are examined in this chapter.

PKC α – Classical PKCs



PKC ζ – Atypical PKCs

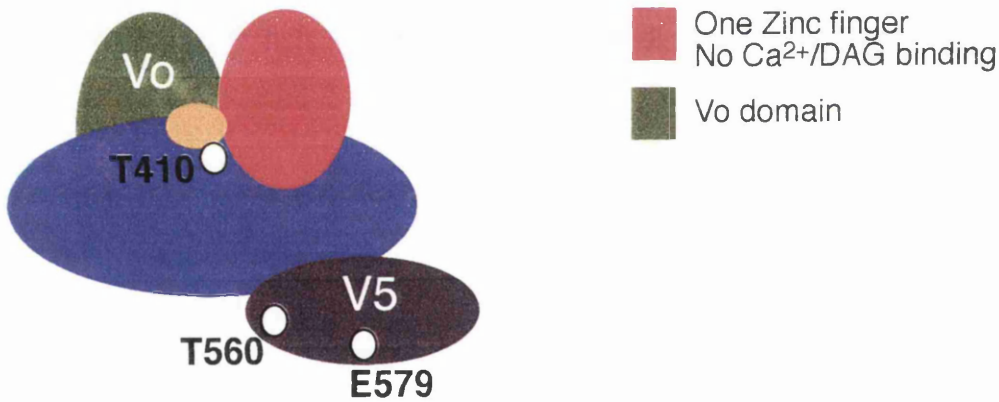


Fig 4.2
Phosphorylation sites in PKC α and the corresponding predicted PKC ζ phosphorylation sites

4.2 Analysis of the Atypical PKC Phosphorylation sites

To help characterise the aPKC phosphorylation sites, anti-sera were raised to the priming phosphorylation sites of PKC ζ. Rabbits were immunised with phospho-peptides (Figure 4.3).

To assess if the equivalent of the known PKC α phosphorylation sites are also occupied in the atypical PKCs *in vivo*, 293 cells were transfected with various constructs of PKC ζ. The antisera are used together with 0.1μg/ml competing de-phospho peptide (Figure 4.4). The antisera specifically interact with the phosphorylated form of the protein. This can be seen by lack of recognition of the phosphorylation sites in the T410E/T560E mutant (EE), where these sites cannot be phosphorylated. The antibodies do not recognise the peptide sequence, only the phosphorylation site. Incubation with the phospho-form of the peptide completely removes the signal. The level of phosphorylation as a percentage of all of the PKC ζ protein population cannot be determined, as the antisera are not titred. The antisera pick up endogenous PKC ζ very faintly, as seen by transfection of the vector alone.

Under normal growth conditions, in the presence of 10% FCS, wild type PKC ζ is phosphorylated in both the activation loop and TP sites. This could be assumed to be a basal level of phosphorylation. The kinase domain construct appears to be equally phosphorylated, in comparison to the wild-type protein. This would suggest that phosphorylation does not appear to be inhibited by the regulatory domain. However, in a cellular environment, the kinase domain may indeed be more highly phosphorylated than the wild-type protein but then be less protected from phosphatases and so phosphorylation sites may subsequently be more readily removed. Furthermore, phosphorylation levels do not appear to be increased by the removal of the pseudosubstrate (PSS) domain, (shown in PKC ζ Δ PSS) a theoretically constitutively active conformation of PKC ζ. Therefore, either the activation loop site is always readily accessible or removal of the PSS site from the catalytic cleft enables increased sensitivity to phosphatases.

How does the phosphorylation state of PKC ζ correlate with activity? To attempt to address this issue, the activities of various PKC ζ constructs were measured. Myc-tagged PKC ζ constructs were expressed in 293 cells and protein immunoprecipitated and eluted. Activity was measured by a kinase

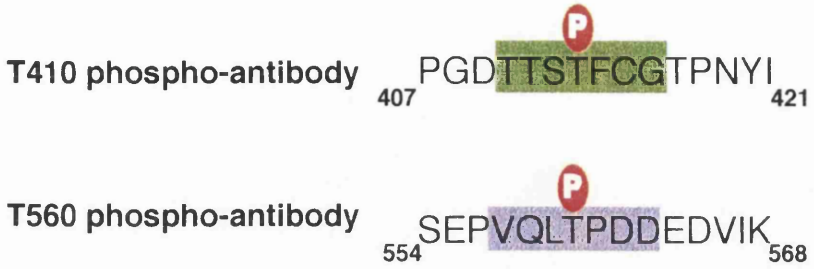


Fig 4.3
Generation of PKC ζ phospho-specific antibodies
The anti-sera recognise the highlighted phosphorylated regions in the activation loop, T410, and TP site, T560.

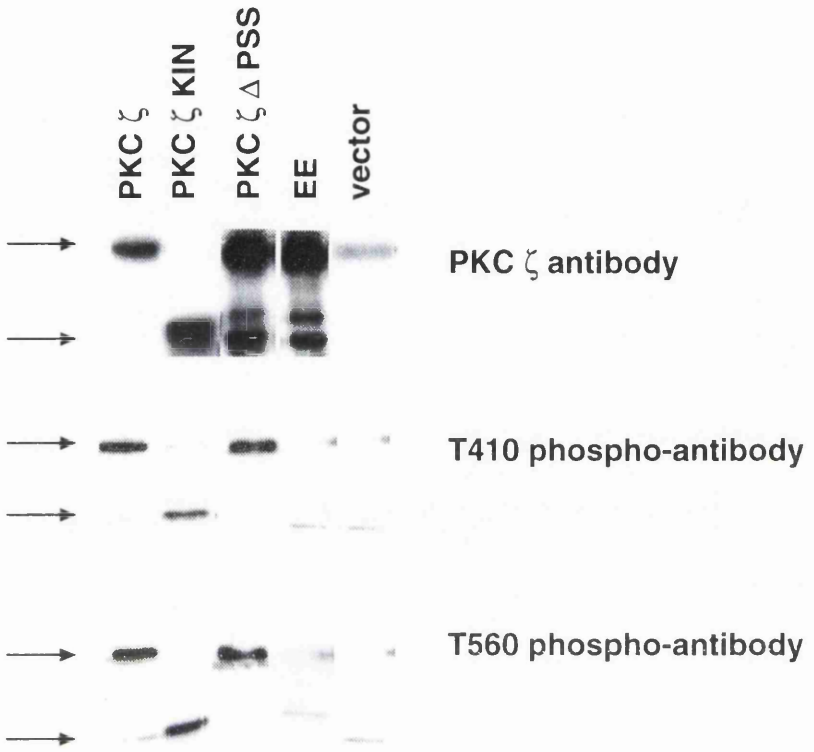


Fig 4.4
Characterisation of the phospho-specific antibodies for PKC ζ
Various PKC ζ constructs were transfected into 293 cells, maintained in 10% FCS and harvested into lysis buffer. Samples were analysed by SDS-PAGE and Westerns carried out using anti-PKC ζ and the phospho-specific antibodies.

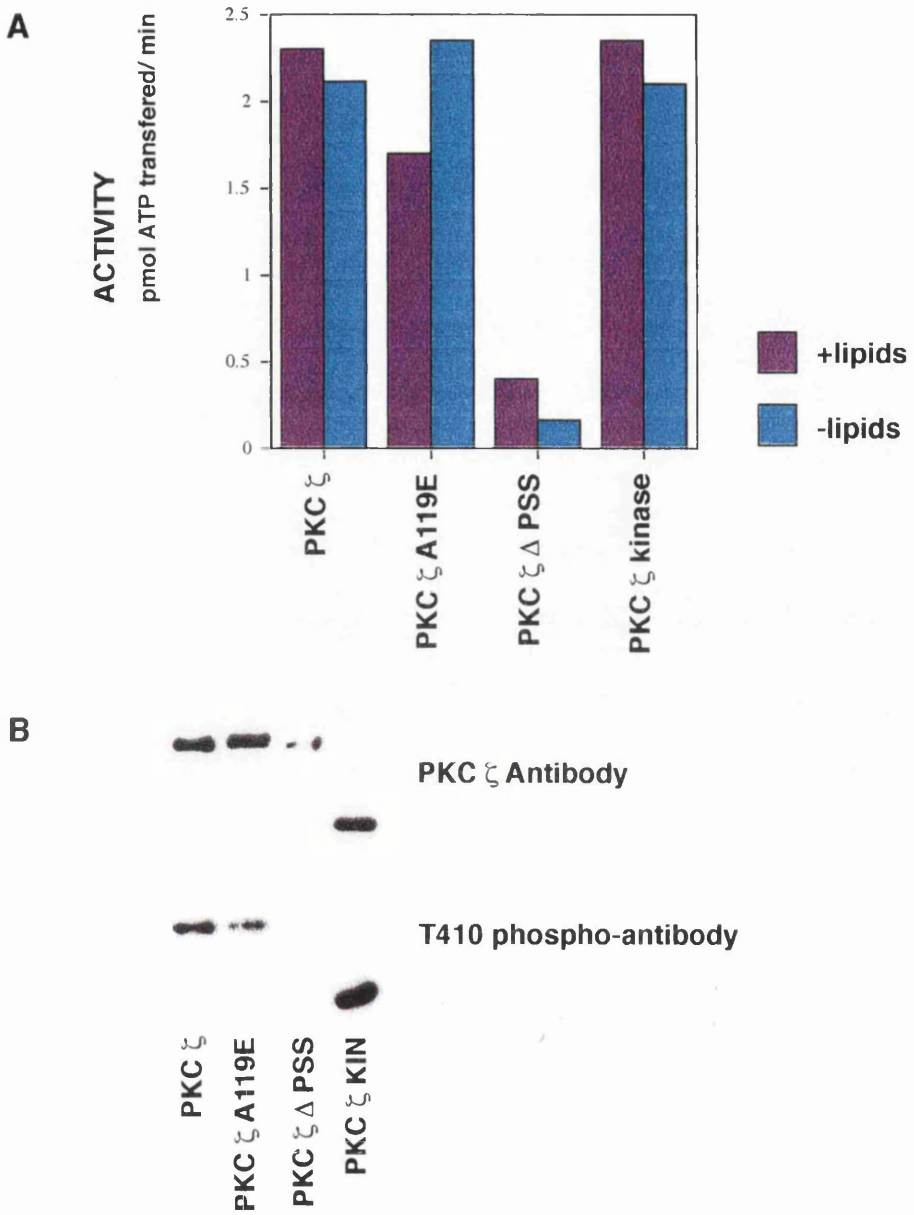


Fig 4.5
Activity of different PKC ζ constructs
293 cells were transfected with various PKC ζ constructs and protein immunoprecipitated and eluted. Kinase activity was measured against phosphorylation of the PKC ζ PSS peptide in the presence or absence of mixed brain lipids (Fig 4.5A). The levels of protein used per assay are shown below (Fig 4.5B), the phosphorylation state of the PKC ζ T410 site was also measured using the phospho-specific antibody.

assay, by monitoring phosphorylation of the PKC ζ PSS peptide or MBP in the presence of ^{32}P γ-ATP and magnesium (Figure 4.5). The choice of substrate did not affect phosphorylation levels and relative activity is the same for both substrates.

The isolated wild-type protein is as active as the constitutively active protein (low expression on elution of the PKC ζ Δ PSS construct in this and other experiments explains the low kinase activity). This supports the phosphorylation site data where, in particular, activation loop phosphorylation (correlating with catalytic competency in PKC α) appears similar in all constructs. The activity of the kinase domain construct is puzzling and appears to vary with preparations of protein. In this case, activity is as high as for wild-type protein. However, on other occasions, the kinase domain (even if expressed and phosphorylated in the activation loop site) is inactive. This would suggest an *in vitro* stability problem. It would be expected that the kinase domain alone acts as a constitutively active kinase, as has been previously demonstrated for PKC ζ (Berra et al., 1993).

From this data, it would appear that wild type PKC ζ is the most active form of the enzyme, within the constraints of the *in vitro* assay system. The amounts of protein isolated from cells are not large enough to be measured quantitatively. This puts a constraint on determining the relative specific activity of enzymes under certain conditions.

4.3 Expression of Bacterial PKC ζ

To try to distinguish between the two phosphorylation sites and to what extent each contributes to activation, bacterial systems which do not encode Ser/Thr kinases that can modify any eukaryotic proteins, were used. Specifically, studies in bacteria could help assess if T560 is an autophosphorylation site. Knowledge from previous expression of PKC α in bacteria showed that unphosphorylated protein is formed which is insoluble in bacteria. Since the most C-terminal 'FSY' site is crucial for regulating protein stability and determines the 'on' rate for phosphorylation, it could be hypothesised that for PKC ζ the glutamic acid in the equivalent position would act to stabilise the protein.

PKC ζ is expressed in bacteria, (Figure 4.6). However, the amounts isolated are variable and high yields are not obtained. The full length protein is very unstable. All possible steps were taken to prevent protein degradation. Cultures were grown at low temperatures and induced at high cell densities

using low (100μM) IPTG concentrations, to allow slow and so not too much protein production. The promoter in the pRSET vector is leaky and shows that induction does not necessarily increase protein yield. Processing occurred at 4°C and the lysozyme method to lyse the bacteria was used, a gentler method than sonication. Bacterial strains containing pLYS and GRoEL/GRoES were also used to ease protein extraction and attempt to improve folding of the protein. Extraction buffers all contained protease inhibitors (see Materials and Methods). Protein was isolated as quickly as possible and stored in 50% ethylene glycol. With these precautions, a crude activity assay was carried out on protein eluted from nickel agarose beads. Protein levels were so low as to make concentration levels difficult to determine, but PKC ζ does show activity above a background vector control level (Figure 4.7).

Bacterially expressed PKC ζ is active, but how this value correlates with mammalian expressed PKC ζ could not be assessed. Bacterial PKC α has an activity of <2% of that of mammalian expressed PKC α (Cazaubon et al., 1994). Bacterial PKC δ is at least 90% less active than COS cell purified PKC δ (unpublished data). This may not be surprising, given that there is unlikely to be a bacterial PKC activation loop kinase to phosphorylate this site. Attempts were made to determine the occupancy of the phosphorylation sites by using Western blotting and phospho-antisera (Figure 4.7). Unfortunately, no success was achieved. The antibodies are not good enough to detect such low levels of protein and non-specifically detect background antigens, seen in the vector control lane. Therefore, it was not determined which sites are occupied and how this relates to activity. Increased amounts of bacterial PKC ζ have to be produced for further analysis.

Bacterially expressed protein will be useful in assessing relative levels of phosphorylation. It would be interesting to see if the TP site is occupied, to indicate whether this is an autophosphorylation site and hence help understand the molecular mechanisms of activation. To date, no further progress has been made with bacterial expressed protein.

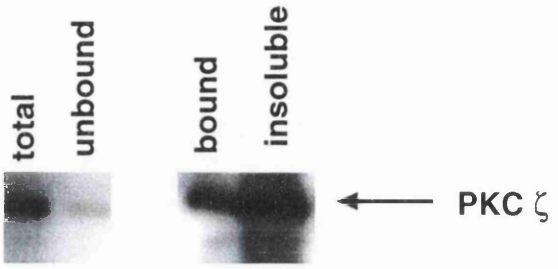


Fig 4.6
Bacterially Expressed Protein
Full length His tagged PKC ζ was expressed in bacteria and after induction, was isolated on Ni-agarose beads (bound sample). Quite a high proportion of the protein is insoluble.

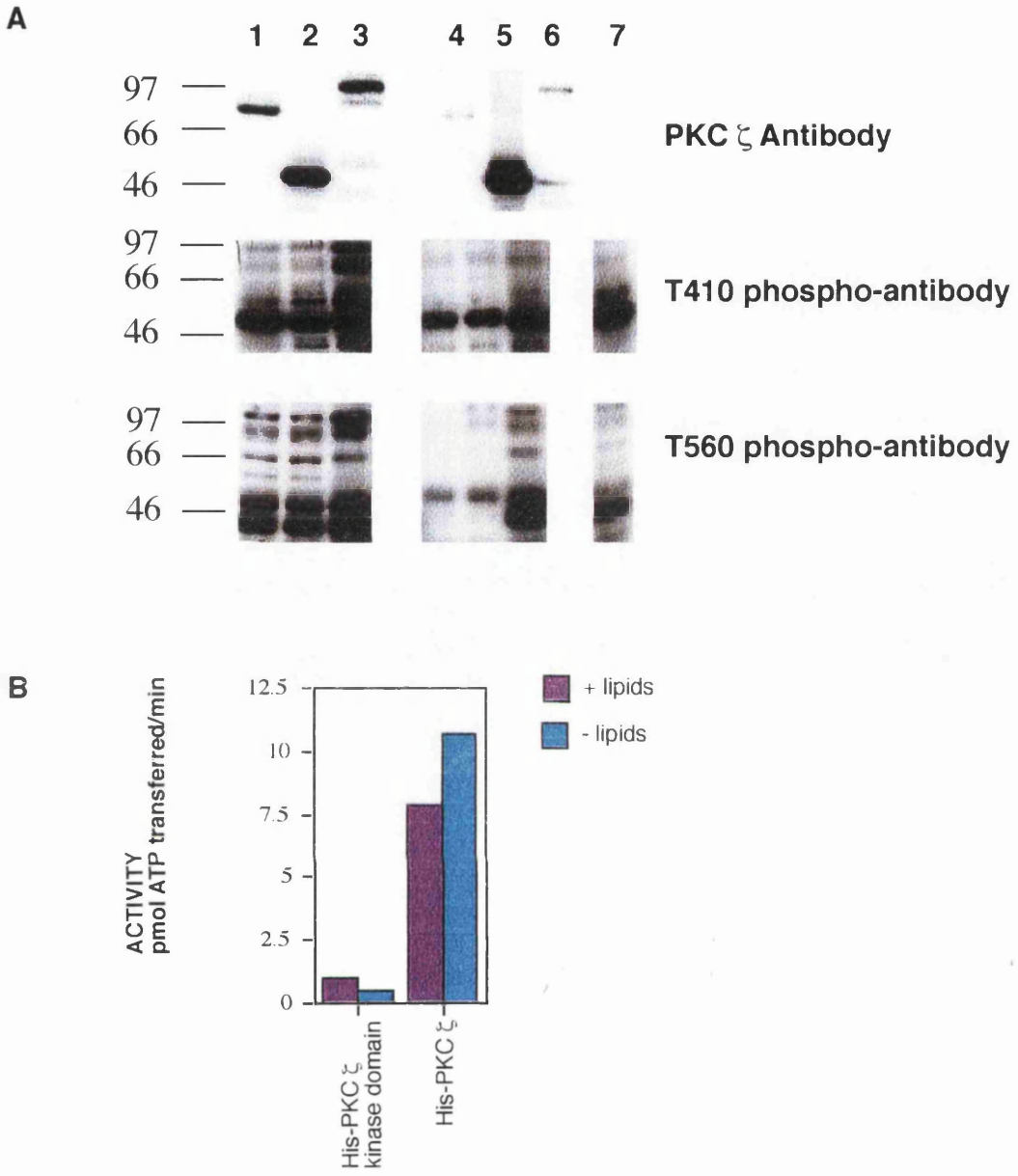


Fig 4.7
Activity of Bacterially Expressed Protein
Various His and GST tagged PKC ζ proteins were expressed in bacteria (Fig 4.7A). The proteins were purified (uninduced or after induction) on Ni-agarose or GST Sepharose beads. Samples were analysed by Western blotting.
Lanes:- 1/4. His-PKC ζ ; 2/5. His-PKC ζ kinase; 3/6. GST PKC ζ 7. vector control
1-3. uninduced, 4-6. 100μM IPTG, 3h induction
The activity of PKC ζ full length (lane 1) and kinase domain (lane 2) were measured in kinase assays using PKC ζ PSS as a peptide (Fig 4.7B).

4.4 Characterisation of Phosphorylation Site Mutants

To elucidate phosphorylation mechanisms and assess which sites are physiologically important, the most widely used technique is the creation of site-directed mutants. Previous mutagenesis studies on PKC α demonstrated that an aspartic acid residue was not as efficient in mimicking phosphorylation due to having a shorter carboxyl side chain (Cazaubon et al., 1994). Mutants were created for PKC ζ (see materials and methods), mutating the TP and activation loop threonine residues into glutamic acids. A previously made activation loop alanine mutant, T410A (Garcia-Paramio et al., 1998) was also analysed.

As has been well documented, phosphorylation cannot be fully mimicked by negatively charged glutamic acid residues. In making the mutants, it is assumed that structural changes induced by the mutations themselves do not affect protein folding or functionality. This was shown to be the case for an autophosphorylation site mutant of PKC δ, S643A. The mutant had reduced activity but still underwent translocation on TPA stimulation and caused 32D myocyte cell differentiation (Li et al., 1997).

All PKC ζ mutants express well in comparison to the wild-type protein in 293 cells (Figure 4.8). The 410E and the EE double mutant appear to express more strongly. The mutant proteins are also cleaved more readily yielding a kinase domain fragment, as is evident in the insoluble fractions. The same has been seen for the ΔPSS site mutant, suggesting that more active conformations of the protein are more readily affected by proteases. Observations of cells transfected with any of the more "activated forms" of PKC ζ indicated a possible role for PKC ζ in cellular attachment. More cells transfected with EE or kinase domain constructs are detached and floating in the media on harvesting. For the EE mutant, this occurs in a concentration-dependent manner. Higher levels of EE DNA transfected is paralleled by increased cell detachment. This is mirrored by immunofluorescence data (see below), where the kinase domain or EE transfected cells result in cell membranes appearing more ruffled. The mutants and the constitutively active form of PKC ζ are all less soluble in Triton-X-100, which further suggests a lesser degree of association with a membrane compartment but instead either localisation with cytoskeletal components or insolubility.

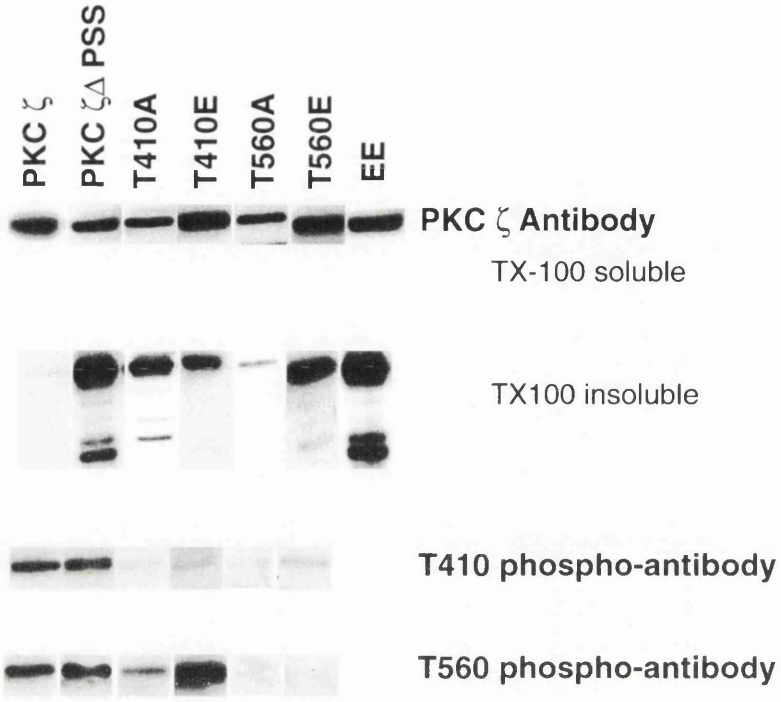


Fig 4.8
Expression and Characterisation of PKC ζ Phosphorylation Site Mutants
293 cells were transfected with different PKC ζ constructs, wild-type or Δ PSS or phosphorylation site mutants. Cells were harvested into lysis buffer and extracts analysed by SDS-PAGE. The Triton-X-100 insoluble fraction was prepared by homogenising the total lysates and centrifuging the samples for 15min at 14 000rpm.

4.5 Molecular Mechanisms involved in PKC ζ Phosphorylation

How do the models proposed so far for the cPKCs apply to the aPKCs? Using phosphorylation site mutants for PKC ζ, how these ideas are related to PKC ζ could be analysed. For PKC ζ, the T410 mutants (A and E) are phosphorylated to a similar extent as wild-type PKC ζ in the T560 site. By comparing the levels of protein expression, there is a slight decrease in T560 phosphorylation in the T410A mutant, however, it is not dramatic. This suggests that the activation loop mutant is of a similar conformation to the wild-type protein, and the TP site is not more exposed on activation loop phosphorylation. If the T410 site phosphorylation reflects the overall PKC ζ activity state, then the T560 site should have increased phosphorylation levels in the T410E mutant. The T560A/E mutants show different patterns of phosphorylation. There is decreased activation loop site phosphorylation in both cases. This may reflect these mutants having a more exposed activation loop site. Thus the T560 site appears to open the protein conformation and expose the protein more strongly to phosphatases.

After elution, the various mutants were assayed for PKC ζ activity (Figure 4.9). T410A is inactive which demonstrates that in PKC ζ this site is essential for catalytic activity. If the protein levels used in the kinase assay are compared, the T410E mutant appears more active than wild-type PKC ζ. However, the T560E and the EE mutants showed low activity or were inactive under these *in vitro* assay conditions. The T560E mutant has low activity which may be due to low occupancy of phosphate in the activation loop site. It would be expected that the EE mutant might be the most active form of the kinase. Moreover in a cellular environment, the EE protein appears to be more active (see the next section), which suggests that the *in vitro* eluted protein is unstable. If cells expressing the T560E or T560A mutant are treated with a phosphatase inhibitor (okadaic acid), there is a significant increase in activation loop phosphorylation (Figure 4.10). This was seen by detection of a slower migrating phospho-form of PKC ζ, using the T410 phospho-antibody. Moreover, incubation of T560E protein with the activation loop kinase (see next chapter), PDK1, increased T410 occupancy and activity, measured by kinase assays. Again, due to relative levels of protein expression, the enzyme is probably then as active as wild-type PKC ζ.

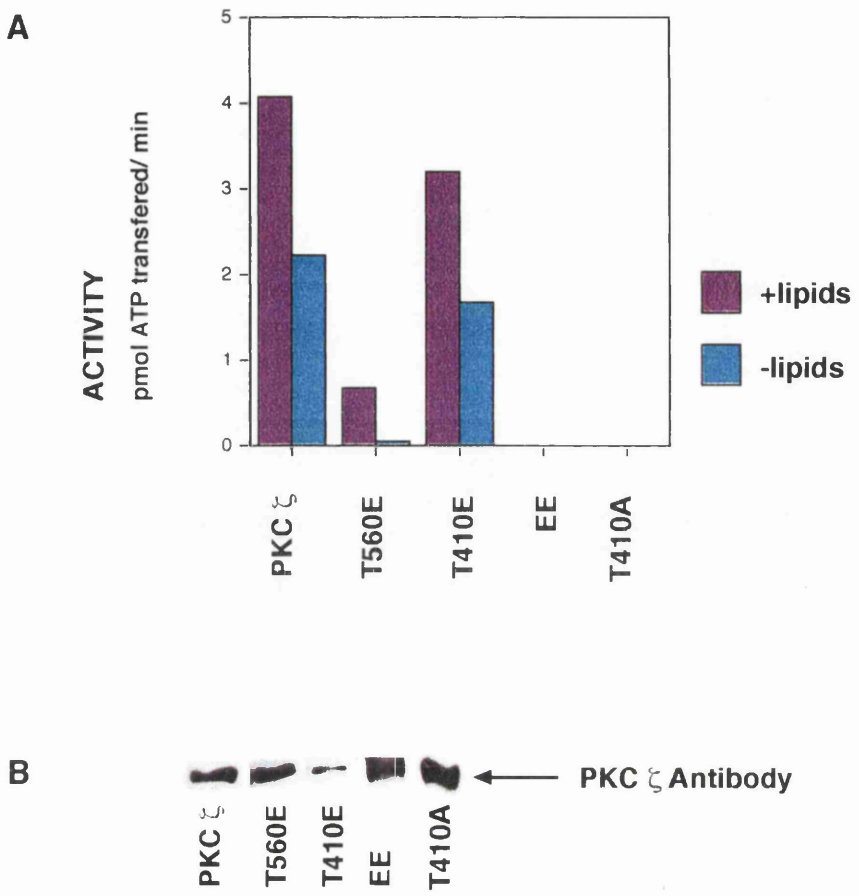


Fig 4.9
Activity of Phosphorylation Site Mutants
The phosphorylation site mutants were expressed in 293 cells and protein Myc-immunoprecipitated and eluted from beads. The activity of eluted protein was assayed using PKC ζ PSS peptide as phosphorylation substrate, in the presence or absence of lipids (Fig 4.9A). The amounts of protein used per assay are shown below (Fig 4.9B).

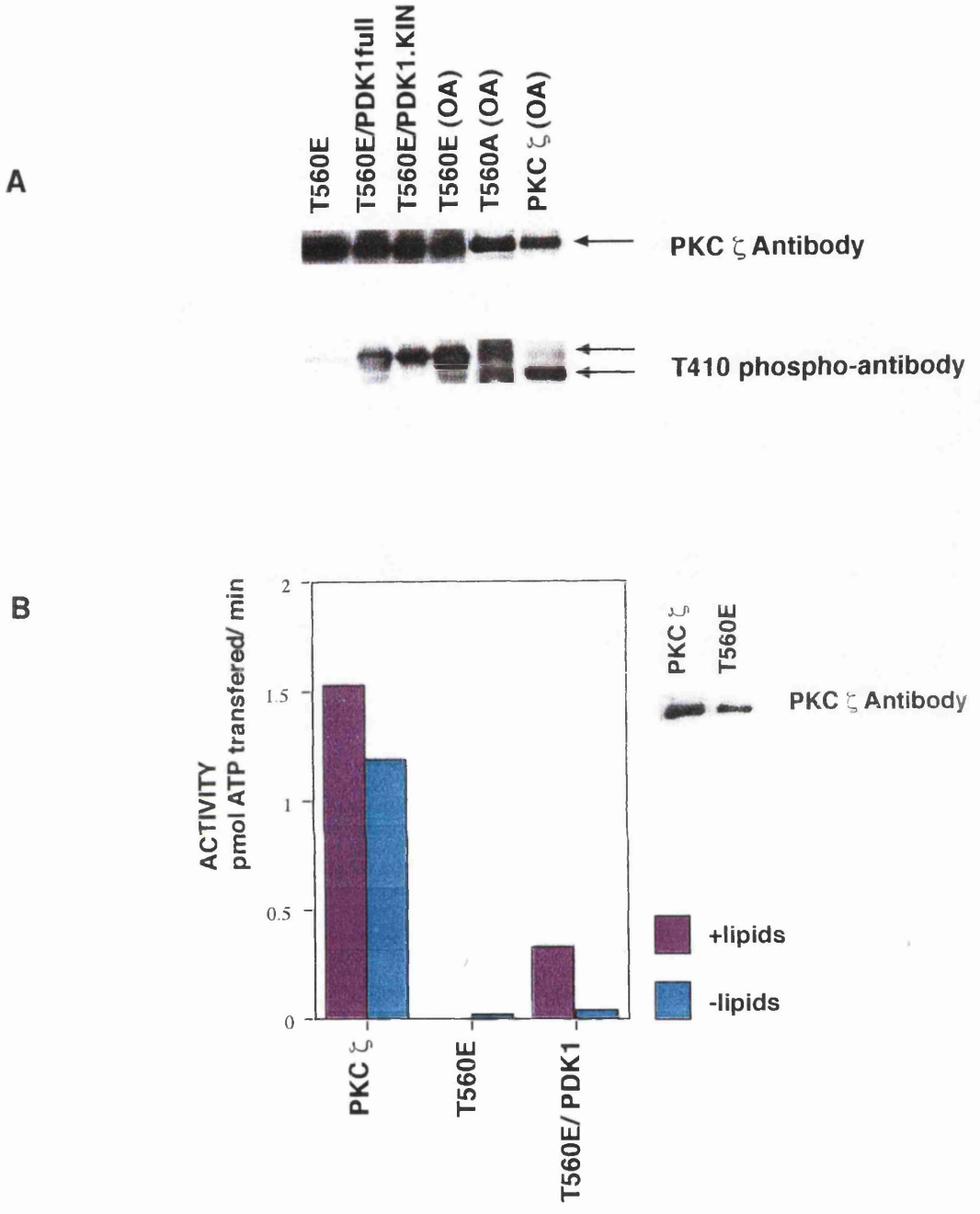


Fig 4.10

PDK1 increases the T560E mutant activation loop phosphorylation and activity

293 cells were transfected with phosphorylation site mutants (T560A or E). Some cells were treated with 500nM okadaic acid (1h). The levels of activation loop site phosphorylation were then measured, using T410 phospho-specific antibodies (Fig 4.10A). The activity of eluted T560E protein after incubation for 30min (with PC/PS and $PI(3,4,5)P_3$) with PDK1 (myc-tagged and eluted from 293 cells) was measured by phosphorylation of PKC ζ PSS peptide in the presence or absence of lipids (Fig 4.10B). Comparative levels of activity of T560E protein and wild-type PKC ζ alone were measured.

4.6 How can the Phosphorylation State of PKC ζ be modulated?

Since the phosphorylation state of PKC ζ seems to reflect its activity, attempts were made to alter levels of PKC ζ phosphorylation and determine which allosteric effectors modulate activation. Cells were treated with several allosteric effectors and documented activators of PKCs or more specifically PKC ζ (Figure 4.11) (ceramides) and the effects on phosphorylation were detected by using the phospho-antibodies.

None of the treatments appeared to have much effect on changing the phosphorylation state of the two sites. DAG and TPA may increase the activation loop site phosphorylation slightly. However, this is unlikely to be a direct effect given the previous literature on DAG/TPA binding (Ways et al., 1992). *In vitro* assays showed that baculoviral expressed PKC ζ was not activated by TPA. Serum starvation does not reduce phosphorylation greatly. Ceramide treatment appeared to increase the TP site phosphorylation, relative to wild-type protein. The only dramatic effect on phosphorylation was seen when PKC ζ transfected cells were incubated with okadaic acid. Clearly the T410 site accumulates phosphate. This implies that a PP1 or PP2A subfamily phosphatase is responsible for dephosphorylating this site. Interestingly, the TP site (T560) is unaffected by the inhibition of an okadaic-acid sensitive phosphatase. This data is important and suggests that PKC ζ can be more highly phosphorylated.

4.7 Characterisation of the Phosphorylation sites in PKC ι

Are the phosphorylation sites and how they are regulated conserved in the aPKC subfamily? The two kinase domains are highly homologous in sequence in the atypical PKCs (Figure 4.12) so it was predicted that the phospho-specific antibodies to PKC ζ would also recognise PKC ι. To verify this, PKC ι was overexpressed in 293 cells and cell extracts blotted with the phospho-antisera.

Clearly the phospho-antibodies detect phosphorylated forms of PKC ι (Figure 4.13) on overexpression and actually detects PKC ι with higher affinity than the C-terminal PKC ι antibody. The occupancy of the sites appears to mirror that of wild-type PKC ζ. Wild-type PKC ι has phosphate in both activation and TP sites. Kinase assays demonstrated that, as for PKC ζ,

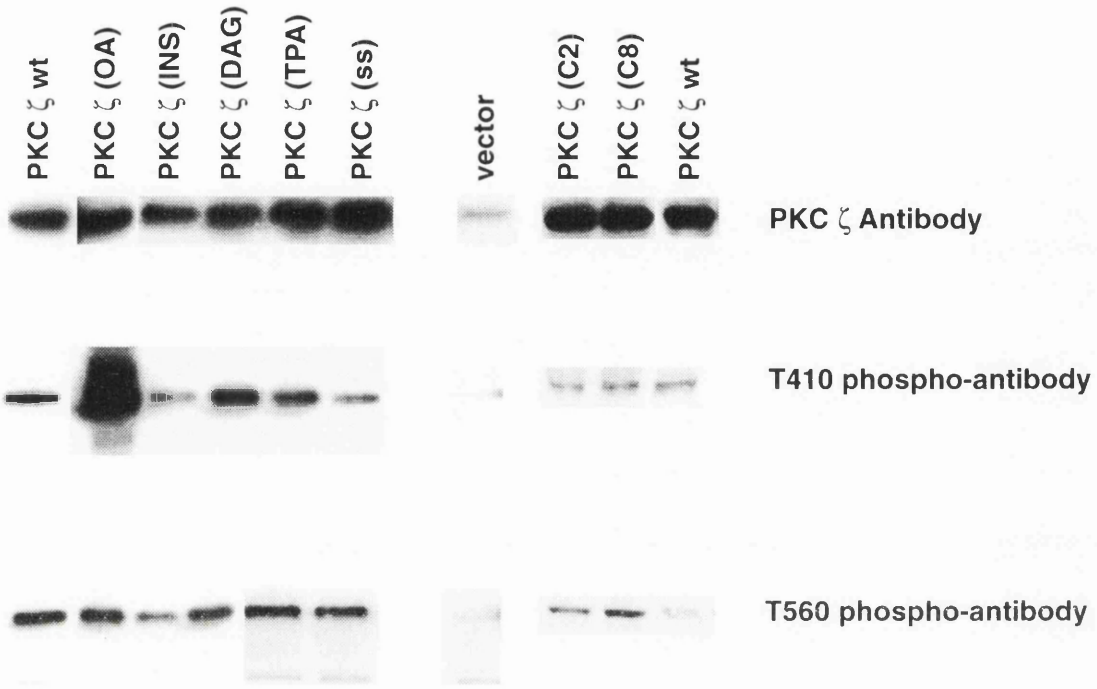


Fig 4.11
Treatment of PKC ζ transfected cells with various agonists to determine the effect on phosphorylation
293 cells transfected with PKC ζ were treated (see below) and then harvested into lysis buffer. Total lysates were analysed by SDS-PAGE.
Treatments were as follows:- OA- 500nM for 1h, Insulin 50μg/ml , DAG - 100μg/μl for 20min, TPA - 400nM for 15min, cells were serum starved for 24h, ceramide - 100μM for 20min

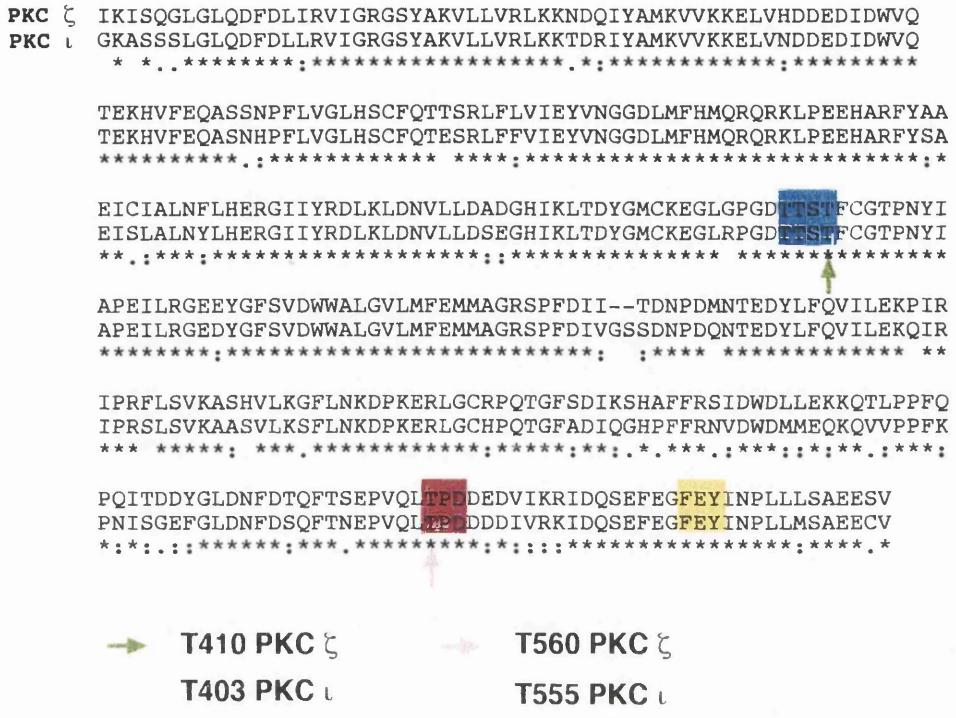


Fig 4.12
Alignment of the Phosphorylation sites in the Atypical PKCs

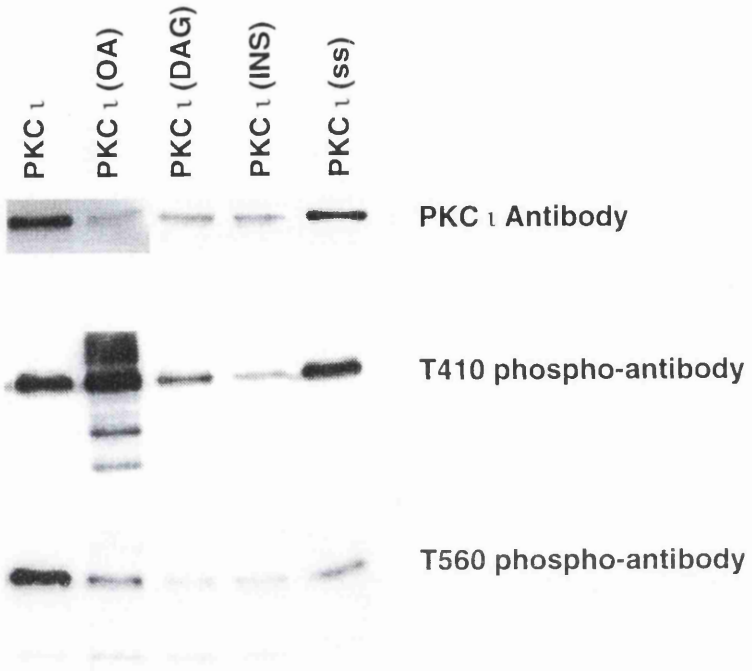


Fig 4.13
Phosphorylation Sites of PKC ι
Cells were transfected with PKC ι and then treated under various conditions (see Fig 4.11). Total lysates were analysed by SDS-PAGE.

PKC ι is active in serum maintained cells. How much further PKC ι can be activated was not investigated. The role of the insulin is contrary to expectation. PKC ι has been documented as being activated by PI3K (Akimoto et al., 1996). Thus insulin should potentially increase phosphorylation. These results may reflect a slow turnover rate of PKC ι phosphorylation. Similarly to PKC ζ, the only extensive accumulation of phosphate in the activation loop site occurred on treatment with okadaic acid. Thus the PKC ι activation loop site is sensitive to PP1 or PP2A phosphatases. As observed for PKC ζ, the TP site is insensitive to these phosphatases. The two atypical PKCs appear to behave similarly in response to various cellular treatments.

4.8 Determining the Properties of the Phosphorylation Site mutants

The physiological role of the PKC ζ phosphorylation site mutants was examined in two ways. Firstly by looking at the effects of the mutants on PKC δ and secondly, by studying their cellular localisation by immunofluorescence. Since the aPKCs do not require the "FSY" site kinase, it is possible that they have specifically evolved the role of phosphorylating the other PKCs in this hydrophobic site. It is known that activation loop site mutants act in a heterogeneous manner and inhibit phosphorylation of other PKCs, not just their own subfamily members (Garcia-Paramio et al., 1998). The activation loop site mutants of PKC ζ inhibited PKC α phosphorylation greatly. From these observations, initial experiments were carried out to determine if other PKCs affected phosphorylation levels of PKC ζ. No differences were observed, however, interesting effects were noted on PKC δ phosphorylation when expressed with PKC ζ. PKC δ activation loop (T505) and FSY site (S662) phosphorylation is increased on TPA stimulation. However, the PKC ζ EE mutant further increased FSY site phosphorylation in a TPA-dependent manner (Figure 4.14A). Moreover, when the T410A inactive PKC ζ construct is expressed with PKC δ, this increase in FSY site phosphorylation is not observed (Figure 4.14B). This shows that the EE mutant is active and suggests that PKC ζ (directly or indirectly) phosphorylates this site *in vivo*.

PKC ζ associates with PKC α or PKC δ weakly (Figure 4.15) in a TPA-dependent manner on co-immunoprecipitation. Whether this correlates with

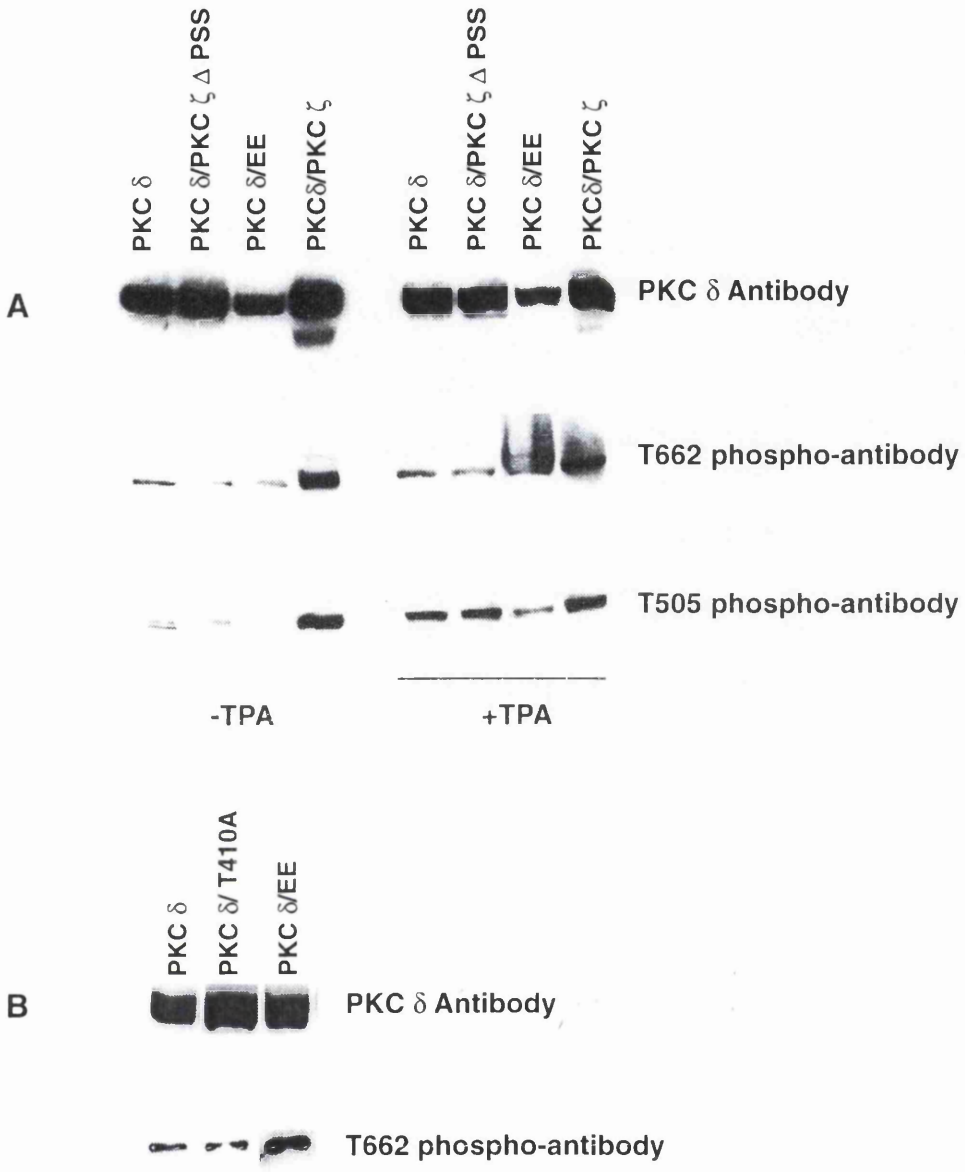


Fig 4.14
The phosphorylation state of PKC δ, co-expressed with PKC ζ and PKC ζ phosphorylation site mutants
293 cells were transfected with PKC δ and either wild-type PKC ζ or constitutively active PKC ζ. Cells were treated with 400nM TPA for 20min or left untreated and then samples harvested into lysis buffer. The levels of PKC δ phosphorylation were analysed by SDS-PAGE (Fig 4.14A). The effects of the PKC ζ T410A mutant on PKC δ phosphorylation were also analysed (Fig 4.14B).

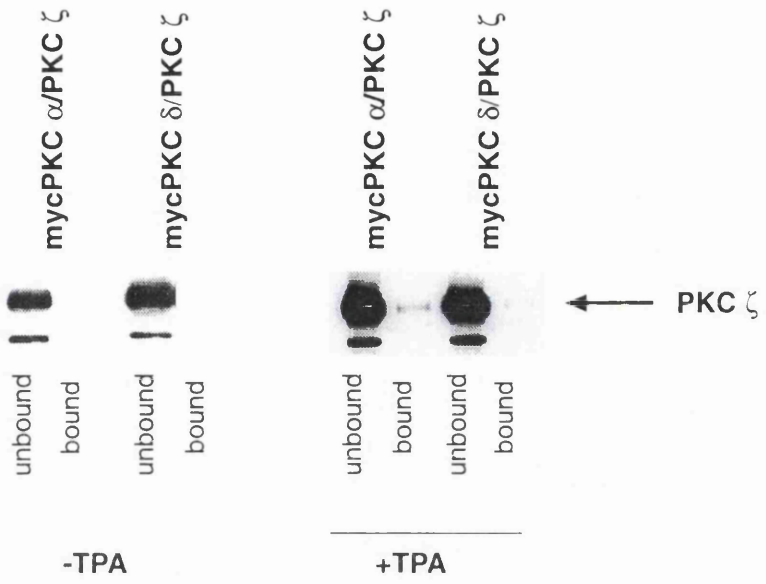


Fig 4.15
Complexing of PKC isoforms
293 cells were transfected with either myc-tagged PKC α or PKC δ, co-expressed with PKC ζ. Cells were treated with TPA (400nM for 15min) or left untreated before immunoprecipitating the myc-tagged protein. Any PKC ζ bound to the precipitated protein was analysed by Western blotting.

Fig 4.16

Cellular Localisation of the PKC ζ phosphorylation site mutants

293 cells were seeded on collagen-coated coverslips and transfected with myc-tagged PKC ζ phosphorylation site mutants. PKC ζ constructs were visualised by staining with a secondary FITC-coniugated antibody. Actin was stained with phalloidin.

Lanes:- A. T410A, B. T410E, C. EE, D. T560E, E. wild-type PKC ζ

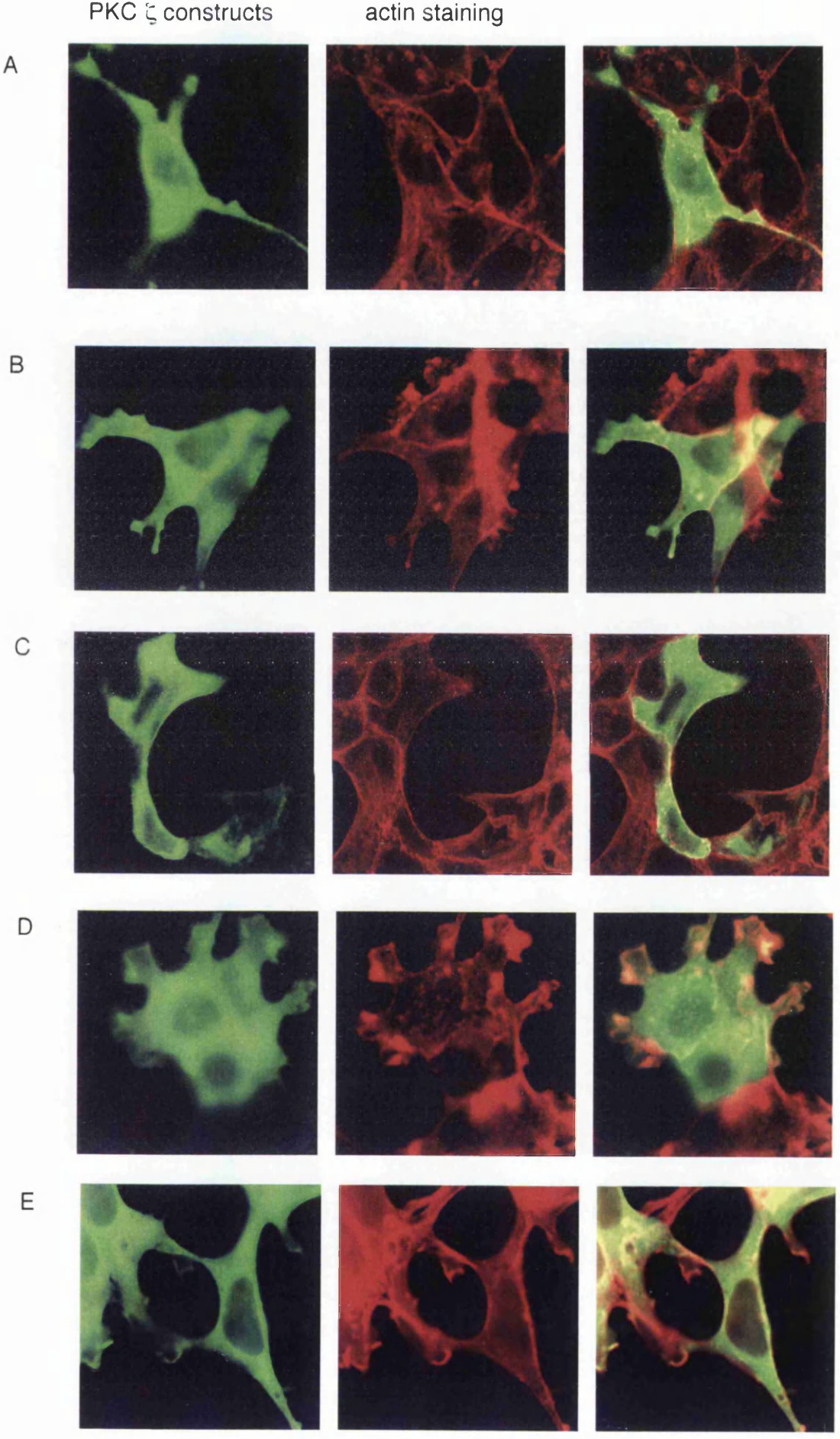


Fig 4.16

PKC ζ being the FSY site kinase or just being associated with activated PKC α or PKC δ has yet to be determined.

4.9 Immunofluorescence

The phosphorylation site mutants appear to be cytosolic in localisation (Figure 4.16). Cells transfected with Ala mutants appear somewhat unhealthy. The TP site mutant (T560E) appears to give cells pseudopodia. T410E transfected cells have ruffled membranes, however, such effects may not be exclusive to transfected cells. PKC ζ may initiate an autocrine response, which would explain these effects. The EE transfected cells detach readily from collagen-coated coverslips and ruffled membranes were observed. Clearly, PKC ζ mediates cytoskeletal organisation but not by direct colocalisation with actin. It will be interesting to investigate which cytoskeletal components are downstream of PKC ζ.

4.10 Discussion

In this chapter, several different tools have been developed to help to elucidate the role of phosphorylation in the activation of aPKCs. Firstly phospho-specific antibodies have enabled direct analysis of the phosphorylation state of PKC ζ in a cellular environment. Secondly, phosphorylation site-specific mutants have furthered the understanding of how the phosphorylation sites correlate with protein activity and stability.

Clearly, aPKCs can undergo phosphorylation in the two conserved priming sites. The activation loop and T560 sites are phosphorylated in active PKC ζ. PKC ζ phosphorylation is unaffected by serum starvation. However, the extensive accumulation of phosphate in the activation loop site on OA treatment demonstrates the potential for greatly increased phosphorylation in the T410 site, possibly by 10-fold. No stimulus tried here resulted in dramatic increases in T560 site phosphorylation.

This chapter pinpoints technical difficulties in studying PKC ζ activity *in vitro*. The recovery of activity is not consistent for particular PKC ζ constructs. This makes it impossible to rigorously compare activity and phosphorylation. The stability of the phosphorylation site mutants *in vitro* is also problematic. Whilst being inactive in *in vitro* assays, the phosphorylation site mutants have been shown to be active in cells, as demonstrated by phosphorylating or indirectly mediating PKC δ phosphorylation, in the FSY site. An "in cell" kinase assay or

cellular readout of PKC ζ activity is required, without too many cellular manipulations, to answer several specific activity questions. However, as is shown in the following chapter, T410 site phosphorylation does result in activation. Therefore, monitoring T410 phosphorylation levels does reflect activity. By being able to view phosphorylation levels by Western analysis and correlate this to activity, this will make a straightforward assay for the activation state of the protein. None of the agonists tested resulted in an increase in T410 site phosphorylation of either PKC ι or PKC ζ. Thus, no acute activation was observed. This is particularly surprising for insulin, given the documented activation of PKC ζ by insulin (Liu et al., 1998; Mendez et al., 1997). However, having the tools to determine the phosphorylation state of aPKCs under various stimuli, will help elucidate upstream activators and identify aPKC-mediated signalling pathways.

Looking at the mechanistic details of PKC ζ phosphorylation, the phosphorylation site mutants demonstrate a co-operativity between the activation loop and TP sites. What role the T560 site plays in activation is unclear. Is it an autophosphorylation site which mirrors T410 phosphorylation? The increase in activation loop site phosphorylation on OA treatment is not mirrored by an increase in phosphate incorporation in the TP site. The bacterial protein expression will help to answer the autophosphorylation question. It is possible that this site is constitutively phosphorylated under these growth conditions used in these experiments. Alternatively, this site may be regulated by a kinase, since lipid treatments alone failed to result in significant increases in phosphorylation. It will be interesting to determine which phosphatase regulates this site. However, the T560 site may just be basally phosphorylated and never occupied to a greater extent. Therefore, from the mutagenesis studies presented here, it appears that occupation of the T560 site is important for the subsequent phosphorylation of the T410 site.

From the data presented in this chapter, it appears that PKC ζ is unique in comparison to all other PKCs. PKC ζ T410 site phosphorylation is inducible and dynamically regulated by phosphatases. This differs from PKC α, which appears to be constitutively phosphorylated under similar growth conditions. This introduces the interesting question of whether all PKCs are differentially regulated or do they need to follow a similar mechanism of activation? Are phosphorylation events a sub-group dependent phenomena? Even PKC δ appears to be regulated in a different manner to the aPKCs. However, fundamentally, all PKCs require phosphorylation of the priming sites for

activation and stability of the conformer. How this is regulated in detail by other factors (scaffold proteins, lipid inputs, phosphatases) has yet to be clearly elucidated. PKC ζ does not appear to undergo downregulation and degradation, instead regulation of PKC ζ phosphorylation is more acute.

The phosphorylation site mutants of PKC ζ which bypass the as yet unidentified controls on the T410 site (E,EE mutants), have biological and biochemical effects consistent with elevated function. These were observed as firstly, promotion of PKC δ S662 phosphorylation and secondly, morphological effects on the actin cytoskeleton. The role PKC ζ plays in cytoskeletal organisation is unknown, but results here demonstrate that constitutively active forms of PKC ζ result in cell detachment. There is increasing evidence that PKC ζ translocates to or is associated with certain cytoskeletal compartments (Saxon et al., 1994). PKC ζ has been found to be associated with actin (Gomez et al., 1995), tubulin (Garcia-Rocha et al., 1997), microtubules, more specifically, spindle fibres (Lehrich and Forrest, 1994), or associated with cellular adhesion, tight junctions (Dodane and Kachar, 1996; Stuart and Nigam, 1995). Whether PKC ζ regulates cortical actin or the formation of tight junctions is unclear. While of interest, these effects are complicated by the need for transfection. Conditioned media from PKC ζ EE-transfected cells have effects on naive cells (D.Parekh, unpublished data). Such autocrine effects, which have also been observed for Ras (McCarthy et al., 1995), make it very difficult to trace the proximal events.

The dynamics of PKC ζ/ PKC ι activation loop phosphorylation indicate that elucidation of the pathway operating on this site would give more precise insight into upstream regulation. This is elucidated in the next chapter.

Chapter 5

5.1 Introduction

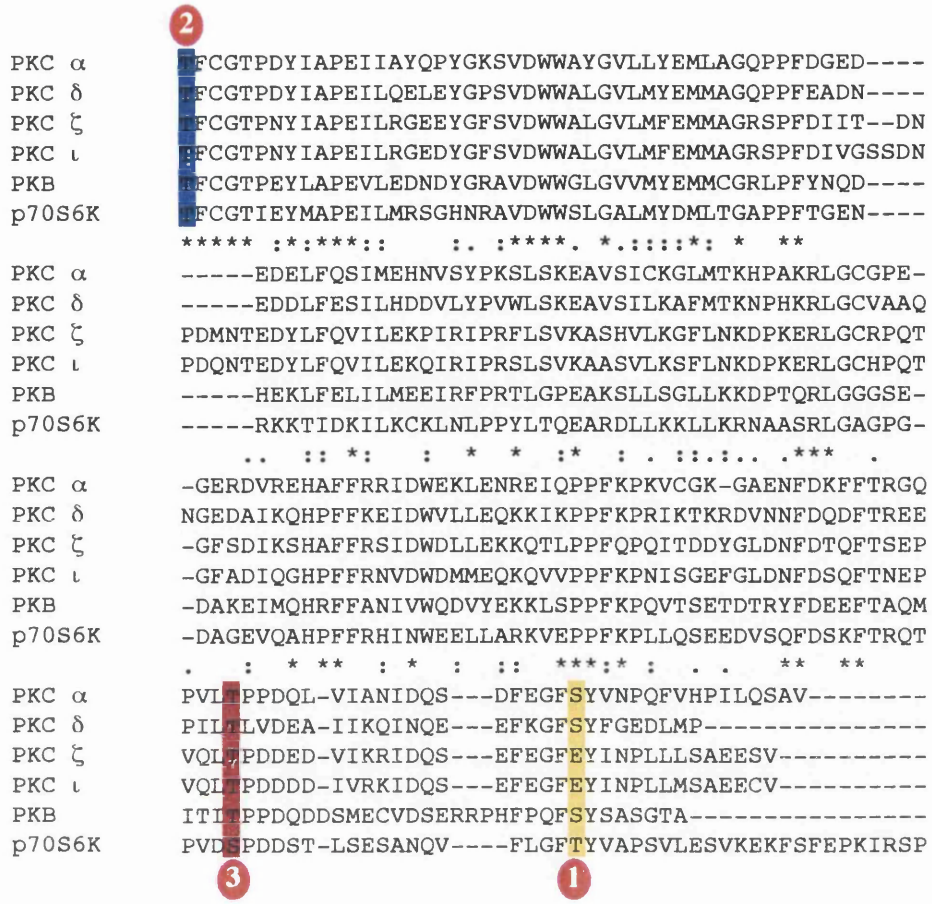
PKB, also referred to as Akt and Rac (Jones et al., 1991), is a Ser/Thr protein kinase which was identified in 1991 by three different groups. Two groups identified the kinase on the basis of its kinase domain homology to PKC (73% similarity to PKC ε) and PKA (68% to the catalytic domain (Coffer and Woodgett, 1991) and at the same time, the kinase was identified as the constitutively active product of an oncogene *v-akt* in rodent T cell lymphomas (a Gag fusion protein attached to cellular Akt, targeting Akt to the membrane (Bellacosa et al., 1991). Several experiments indicated that PKB was regulated by insulin and growth factors (Alessi et al., 1996), i.e. PDGF, NGF, EGF (Franke et al., 1995) and several lines of evidence implicated regulation by PI3K. Firstly, PKB has an amino-terminal PH (pleckstrin homology) domain and the lipid products of PI3K (i.e. $PI(3,4,5)P_3$ and $PI(3,4)P_2$) can bind here (Franke et al., 1997; Frech et al., 1997; Klippel et al., 1997). Whether inositol phospholipids directly regulate PKB activity is still unclear (James et al., 1996). Growth factor induced activation of PKB is inhibited by either chemical inhibitors of PI3K, e.g. wortmannin (Ui et al., 1995) or co-expression with dominant negative forms of PI3K or PDGF receptor mutants, which cannot bind PI3K regulatory subunits. Moreover, in response to insulin and IGF 1, PKB was found to become phosphorylated on two separate sites - T308 (the Thr residue lies in the activation loop site, see earlier descriptions of the kinase domain) and S473 (equivalent to S657 in the hydrophobic region of PKC α. Kinase inactivated mutants were still phosphorylated, which rules out autophosphorylation events. Removal of the phosphates by incubation with phosphatases (*in vitro*) resulted in inactivation of PKB. Thus, these two sites are crucial for PKB activity. The kinase which phosphorylates T308 was identified independently by two different groups. Both groups used cellular lysates from either rat brain (Stephens et al., 1998; Stokoe et al., 1997) or skeletal muscle (Alessi et al., 1997) and purified proteins over a MonoQ column and separated fractions were tested for their ability to phosphorylate PKB *in vitro* in a $PI(3,4,5)P_3$/ATP-dependent manner. The isolated kinase activity was further purified and cloned and termed PDK1 (PIP_3-dependent kinase 1).

PDK1 is a 63kD protein with an amino-terminal kinase domain and a carboxy-terminal PH domain (Alessi et al., 1997). The PH domain is a domain

of about 100 amino acids which has similarities to other conserved PH domain sequences (even though homology at the sequence level is low), including an invariant Trp 535 residue. Secondary structure predictions indicate that it is likely to form β sheet structures and an extended α- helix, consistent with other known PH domain structures (Ponting et al., 1997). The kinase domain possesses several consensus sequences for Ser/Thr kinases, including the DLKPEN and DFG (ATP binding) sites, but interestingly also contains a putative activation loop site motif. Whether this implies that PDK1 dimerises and transphosphorylates itself or that this kinase is under the same phosphatase regulation as its substrates is unclear.

The PDK gene has been mapped to chromosome 16p13.3 (Alessi et al., 1997). Known intron-exon boundary data in this region indicates that this locus gives rise to alternatively or incompletely spliced transcripts. To date, four PDK activities have been identified (Stephens et al., 1998). The isoform described here is the full length protein, cloned by D.Alessi and colleagues. Another isoform is known to be missing the substrate recognition region (amino acids 238-263) and is a kinase dead isoform (Stephens et al., 1998). PDK1 is homologous to a Drosophila protein, DSTPK61 (54% identical in the catalytic domain and 61% in the PH domain). DSTPK61 is differentially spliced and encodes a Ser/Thr kinase, however, its role in signalling has not been established. It plays a role in sexual differentiation, oogenesis and spermatogenesis (Alessi et al., 1997). It will be interesting to see if there is any functional connection in mammalian cells. Elucidation of its role in signalling may give insights into how PDK1 is regulated.

PDK1 phosphorylates and activates PKB α *in vitro* (0.4-0.6 moles of phosphate are incorporated in the presence of PC/PS and $PI(3,4,5)P_3$) and *in vivo* (immunoprecipitated material from transfected PKB and PDK1 in 293 cells shows a 20 fold increase in activity). The site which is phosphorylated was identified by tryptic digestion and phosphopeptide mapping. Analysis shows that PDK1 only phosphorylates T308. The other site known to be sensitive to PI3K regulation is S473, which is not phosphorylated by PDK1 and the activity of a T308A mutant cannot be altered by PDK1.



1 **C-terminal "FSY" site (not for atypical PKCs)**

2 **Activation loop site - PDK1**

3 **TP site**

Fig 5.1
Conservation of the Activation loop Threonine Phosphorylation sites in the AGC Family Members

The role inositol lipids play in PDK1 activity and substrate specificity will be discussed in more detail below.

5.2 Atypical PKCs

Atypical PKCs differ from all other PKCs in that they have a charged amino acid, a glutamic acid, in the most C-terminal phosphorylation site. It has been shown that a negative charge in all other PKCs and ACG kinases, in the form of a phosphate group, is important in this position, for example (Orr and Newton, 1994). This site is believed to be phosphorylated by an as yet unidentified kinase. Whether it is an evolutionary advantage for the atypical PKCs to bypass this kinase and consequently be under less stringent regulation is unknown. However, if you relate all the models we have concerning PKC α, it would imply that the first phosphorylation event for the aPKCs involves activation loop site phosphorylation. Conservation of a number of phosphorylation events in members of the AGC kinase subfamily and activation loop site conservation (Figure 5.1) led us to question whether the recently identified PKB upstream kinase, (PDK1), was responsible for the activation loop phosphorylation of PKC isotypes.

5.3 *In vitro* Activation of PKC ζ

To identify if PKC ζ is a substrate for PDK1, initially *in vitro* experiments were carried out. GST tagged PDK1, purified as described in (Alessi et al., 1997) was incubated together with baculovirus expressed PKC ζ and the activity (measured by incorporation of [^{32}P-γ] ATP) and the activation loop site occupation was monitored using phospho-antibodies.

Figure 5.2A shows that there is clearly an increase in PKC ζ phosphorylation paralleled by an increase in T410 site phosphorylation (Figure 5.2B) when PKC ζ is incubated with PDK1. This suggests PKC ζ is phosphorylated by recombinant PDK1. The maximum stoichiometry of phosphorylation is 2 mol/mol. This represents not only the activation loop site phosphorylation but also phosphate incorporation into autophosphorylation sites in PKC ζ. PKC ζ phosphorylation by PDK1 activated the protein five-six fold, as determined by phosphorylation of a pseudosubstrate site peptide (Figure 5.3). Lipids had little effect on phosphorylation and activation *in vitro*.

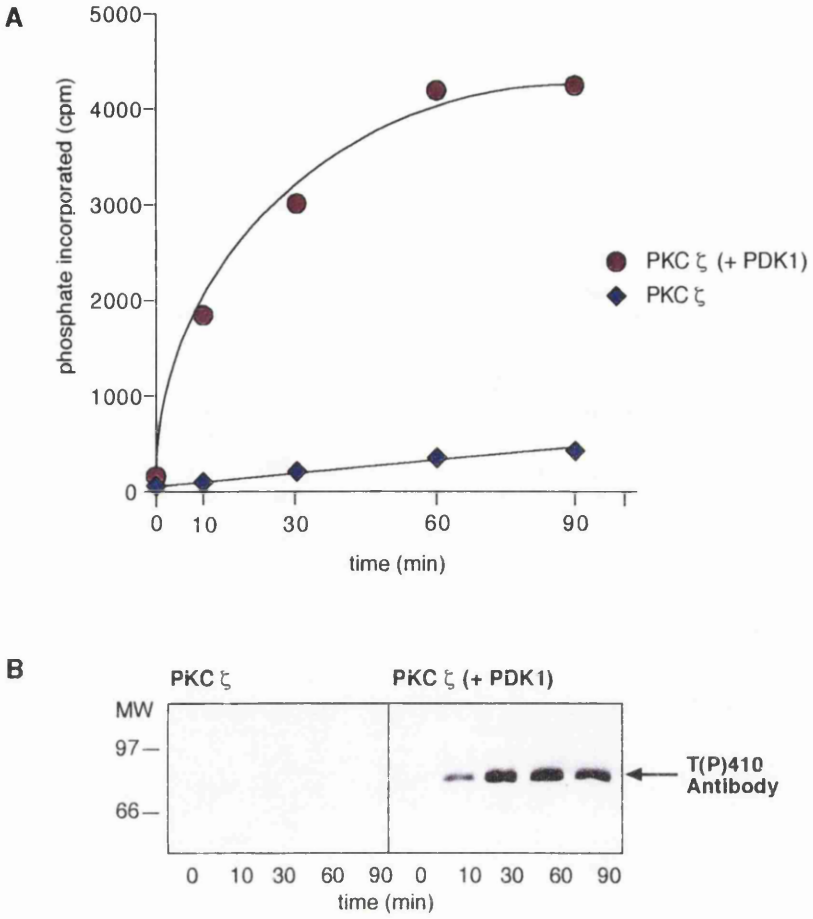


Fig 5.2
***In vitro* phosphorylation of PKC ζ by PDK1**
Incubations were carried out in the presence of 100μM phosphatidylserine (PS), 10μM phosphatidylinositol-3,4,5-phosphate (PIP_3), 100μM phosphatidylcholine (PC) and 0.5 μM phorbol ester (TPA). Incorporation of ^{32}P- ortho-phosphate (Fig 5.2A) enabled determination of the stoichiometry of phosphorylation. Increase in the activation loop (T410) site phosphorylation is measured using phospho-specific antibodies (Fig 5.2B).

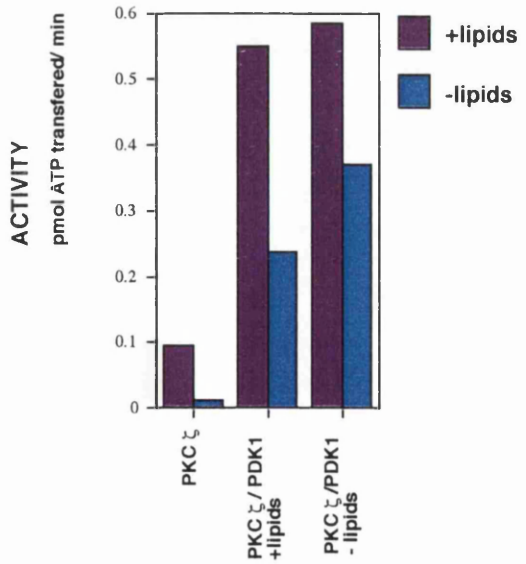


Fig 5.3
PDK1 increases PKC ζ Activity
Activity of PKC ζ was measured by phosphorylation of a pseudosubstrate peptide. PKC ζ and PDK1 were pre-incubated with and without lipids (see Fig 5.2). PKC ζ kinase assay was carried out as described in Materials and Methods.

5.4 *In vivo* Activation of PKC ζ

The data above demonstrates that *in vitro*, PDK1 can act as an activation loop kinase for PKC ζ. To identify if PDK1 can increase PKC ζ phosphorylation *in vivo*, PDK1 and PKC ζ were co-expressed in 293 cells. Figure 5.4 demonstrates that when PDK1 and PKC ζ are co-expressed, PKC ζ phosphorylation in the activation loop site occurs. Removal of the PH domain of PDK1 does not affect its functionality. When the PDK1 kinase domain alone is expressed, T410 site phosphorylation is highest (when PKC ζ is expressed alone, the basal level of T410 phosphorylation is very low, see below).

Several proteins which are substrates for kinases can also bind the kinase. This may be true for PDK1 and PKC ζ. PDK1 may need to recognise and bind to a certain region of PKC ζ before being able to phosphorylate the activation loop site. To address this question, myc tagged PDK1 constructs were expressed with PKC ζ and immunoprecipitated.

Deletions within the PDK1 cDNA reveal that the kinase domain of this protein is not only sufficient but complexes most strongly with PKC ζ (Figure 5.5). The N-terminal deletion of PDK1 does not express as well as the full length construct which explains the reduced PKC ζ recovery. However, removal of neither the 50 N-terminal amino acids nor the C-terminal PH domain influenced co-immunoprecipitation. It is also notable that PDK1 still complexes with PKC ζ when the activation loop site is phosphorylated (Figure 5.5B). This suggests that PDK1 does not distinguish between the phosphorylated and dephosphorylated forms of PKC ζ and indeed phosphorylation does not result in complex dissociation. It seems likely that the binding site is independent of the substrate recognition site.

The following experiments were undertaken to determine where the binding region is in PKC ζ. Various myc-tagged PKC ζ constructs were expressed in 293 cells together with full length (EE tagged) PDK1. No co-immunoprecipitation of PKC ζ with PDK1 was observed for either the Vo or the entire regulatory domains (Figure 5.6). This is interesting since it suggests that the interaction is probably not unique to PKC ζ, since PKCs are most highly conserved in their kinase domains and the question of isoform specificity may be answered by regulatory domain binding proteins. Mutations within the pseudosubstrate site, which have been shown to

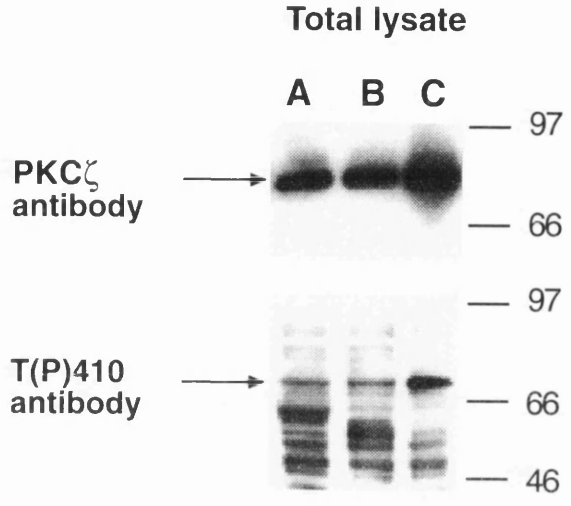


Fig 5.4
***In vivo* Phosphorylation of PKC ζ by PDK1**
PKC ζ was co-expressed with different constructs of PDK1. The protein levels were determined using PKC ζ antibody and phosphorylation levels on total lysates detected using a T410 phospho-specific antibody.
Lanes:- A-full length PDK1; B-PDK (51-556); C-PDK kinase domain (51-404)

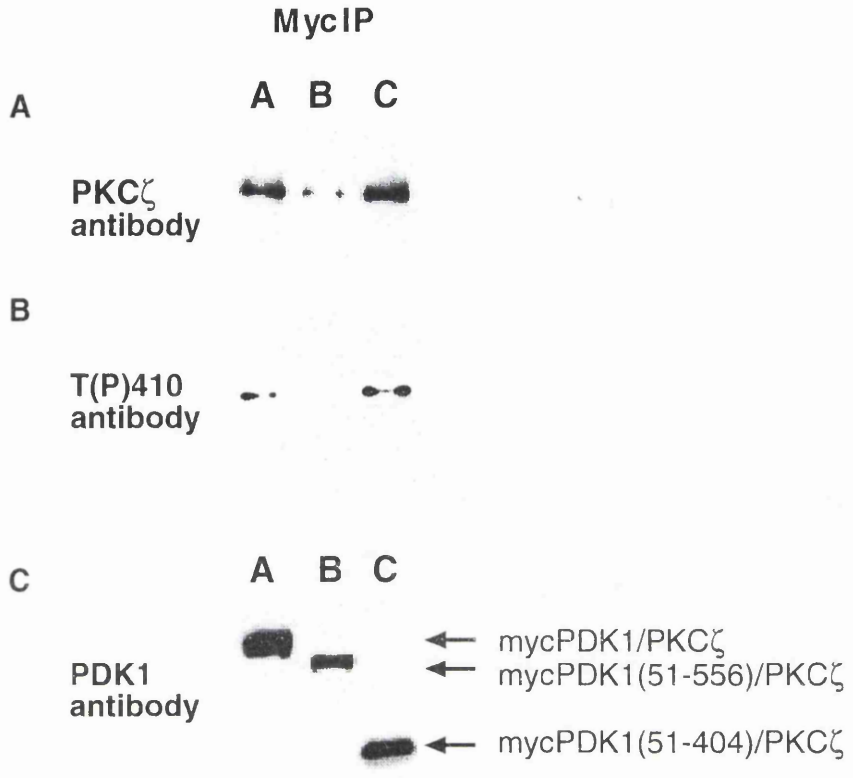


Fig 5.5
PDK1 Complexes with PKC ζ
Several Myc tagged PDK constructs and non tagged PKC ζ were expressed in 293 cells. PDK was immunoprecipitated with a Myc antibody and any bound PKC ζ analysed using the PKC ζ antibody (Fig 5.5A). The phosphorylation state of PKC ζ was visualised using the phospho-specific antibodies (Fig 5.5B).

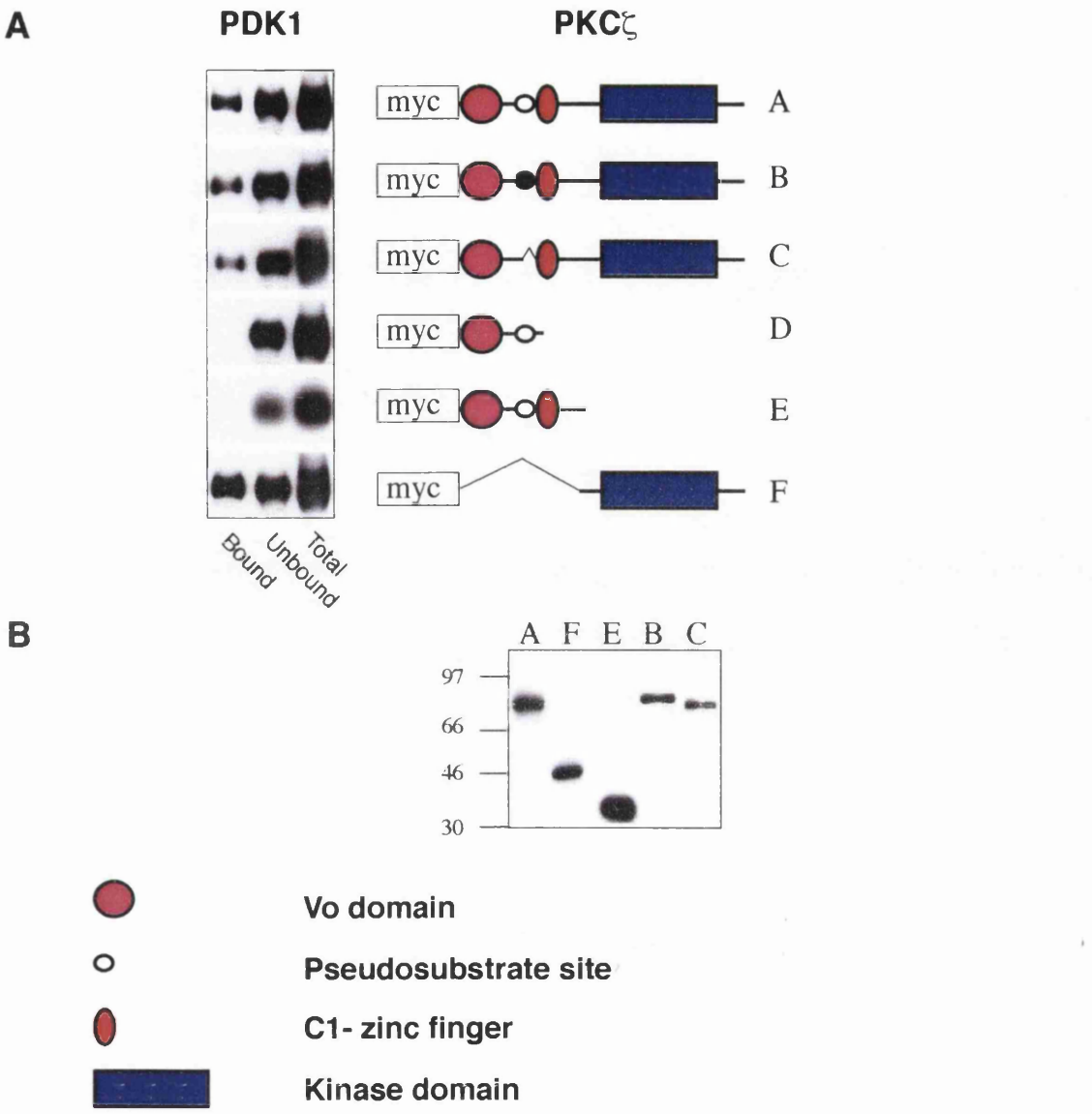


Fig 5.6
PKC ζ interacts with PDK1
Myc-tagged PKC ζ constructs were expressed in 293 cells with EE tagged PDK1. PKC ζ was immunoprecipitated and bound, unbound and total PDK1 detected by immunoblotting (the same cell equivalents were loaded). The levels of PKC ζ immunoprecipitated are shown in Fig 5.6B.

Lanes:- A-full length PKC ζ; B-pseudosubstrate A119E mutant; C-pseudosubstrate deletion Δ 116-122; D-Vo domain (1-135); E-regulatory domain (1-232); F-kinase domain (232-595)

activate PKC ζ and remove the autoinhibition by the PSS on the catalytic site, may have a more exposed activation loop site (Pears et al., 1990). However, this had no influence on the extent of complex formation emphasising that an independent site is recognised by PDK1. The kinase domain alone is sufficient for complex formation between PKC ζ and PDK1. In fact, PKC ζ kinase domain binds the strongest to PDK1 as judged by the extent of recovery relative to unbound PDK1 (the kinase domain is not expressed any more efficiently than other PKC ζ constructs). Thus the kinase domain of PDK1 binds to the kinase domain of PKC ζ and activates PKC ζ by T410 phosphorylation.

Clearly PDK1 and PKC ζ complex together by kinase domain interactions, resulting in activation and an increase of phosphorylation in the T410 site. However, as has been seen in several signalling cascades, for example, in the MAPK cascade (in yeast (Whitmarsh and Davis, 1998) and recently, in mammals (Schaeffer et al., 1998; Whitmarsh et al., 1998), scaffold proteins may be required to bring PDK1 and PKC ζ into contact. PDK1 may possibly require a chaperone protein to recognise PKC ζ. Attempts were made to address this issue and try to identify if any other proteins are bound to the PKC ζ-PDK1 complex. Using ^{35}S methionine immunoprecipitation, various combinations of proteins were expressed in 293 cells:- PDK alone or PKC ζ and PDK1. Any binding proteins were visualised by autoradiography (in comparison to vector alone) (Figure 5.7). No obvious binding partners were identified above background for PDK1 or PKC ζ/PDK1. If myc-tagged PKC ζ is expressed with PDK1 (EE tagged), PDK1 is pulled down, further confirming the interaction. PDK1 expressed with PKC ζ may have a slightly increased molecular weight, suggesting potential phosphorylation and activation of PDK1 by PKC ζ. It is still possible that PKC ζ-PDK1 may recruit other proteins but this may require the presence of a membrane or occur only on stimulation.

To examine in more detail if the PDK1-PKC ζ interaction is affected by the phosphorylation state of PKC ζ, various phosphorylation site mutants of PKC ζ were co-expressed with PDK1 (Figure 5.8). It may be possible that if T560 autophosphorylation follows T410 phosphorylation, this phosphorylation event may cause PDK1 to dissociate from PKC ζ. However, immunoprecipitation experiments suggest that phosphorylation or lack of it in any site does not affect the interaction with PDK1. Thus the PDK1 binding

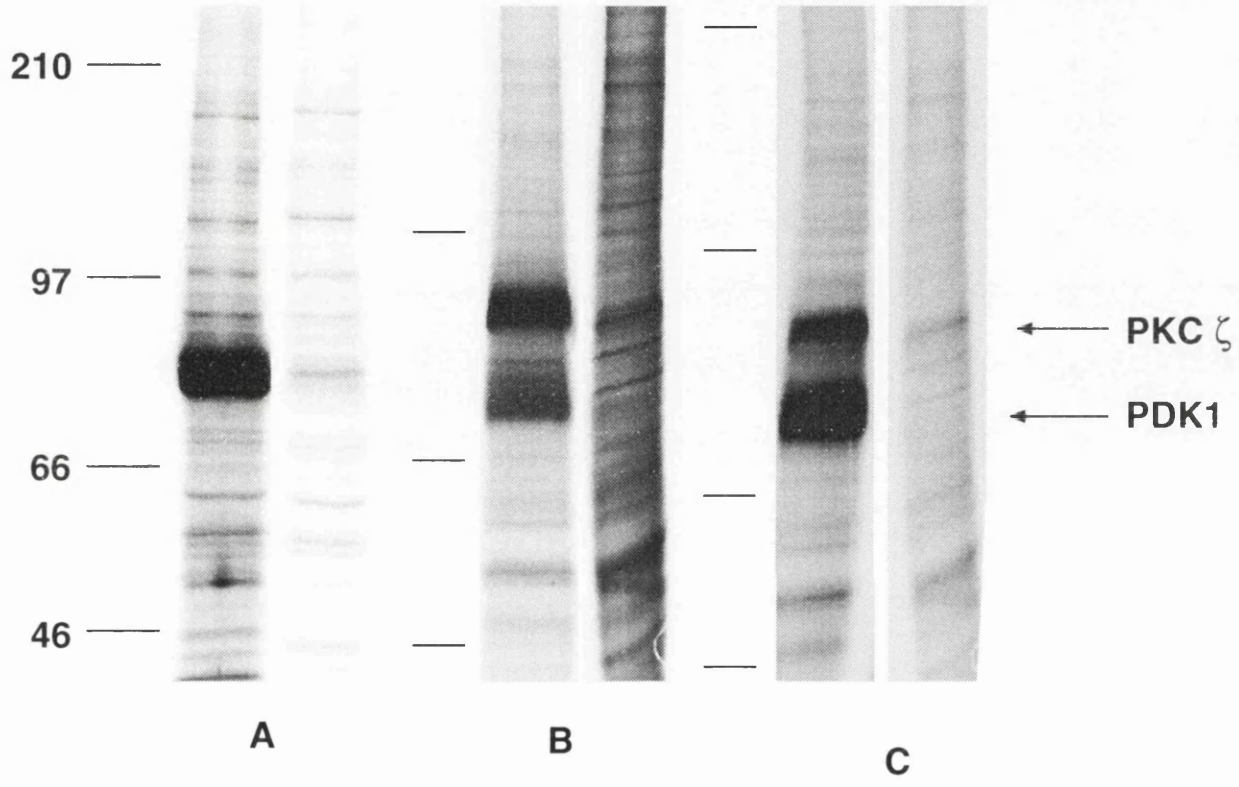


Fig 5.7
The PKC ζ – PDK1 Complex
Myc-tagged PDK1 or PKC ζ were expressed in 293 cells (left hand side) under ^{35}S-methionine labelling conditions (see Materials and Methods) and immunoprecipitated. All proteins were compared to vector controls (right hand side) for each immunoprecipitation experiment.

In Fig 5.7A, myc-tagged PDK1 alone was expressed. In Fig 5.7B, myc-tagged PKC ζ was expressed with untagged PDK1. In Fig 5.7C, myc-tagged PKC ζ and PDK1 were expressed.

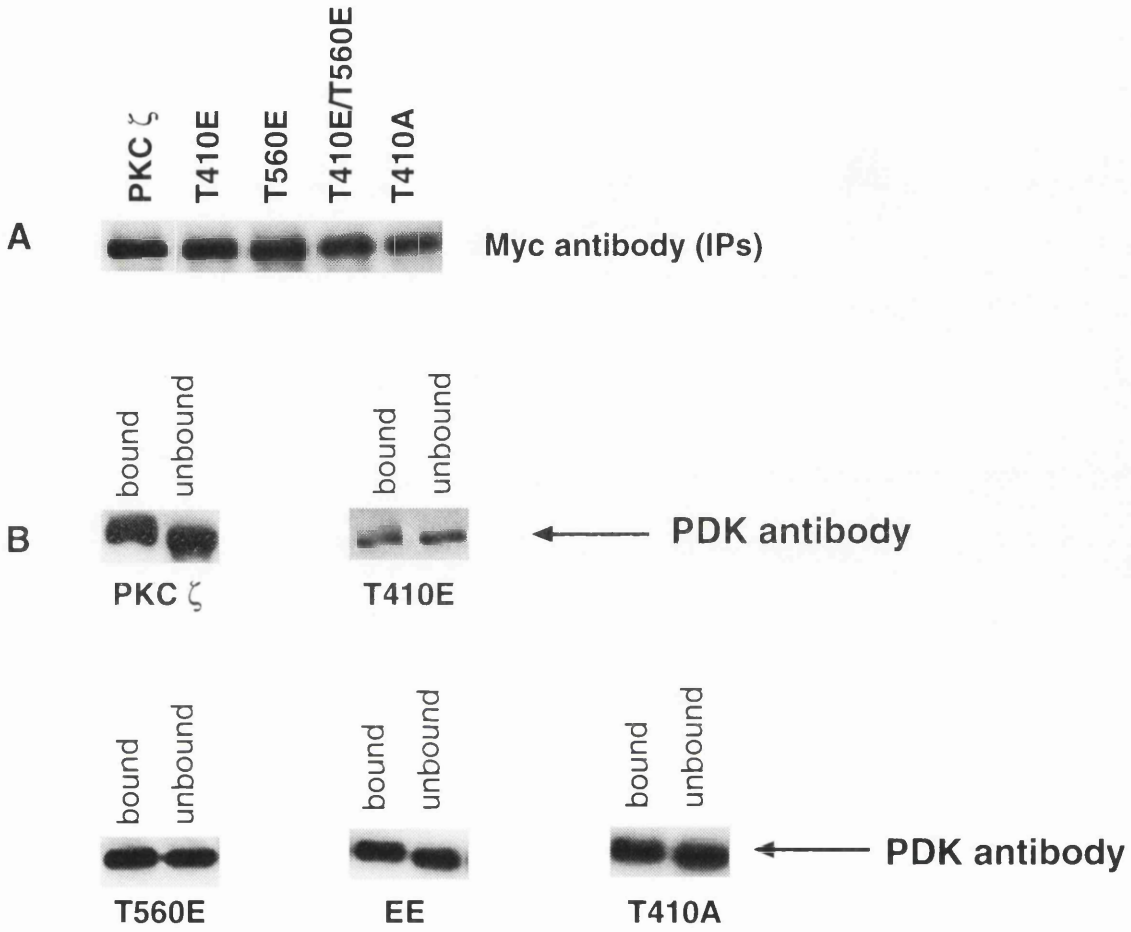


Fig 5.8
PDK1 binds to the Phosphorylation Site Mutants
Several myc-tagged phosphorylation site mutants were expressed with EE-tagged PDK1. Myc tagged proteins were immunoprecipitated and any bound or unbound PDK analysed by Western blotting (Fig 5.8B). Amounts of immunoprecipitated proteins are equal (Fig 5.8A).

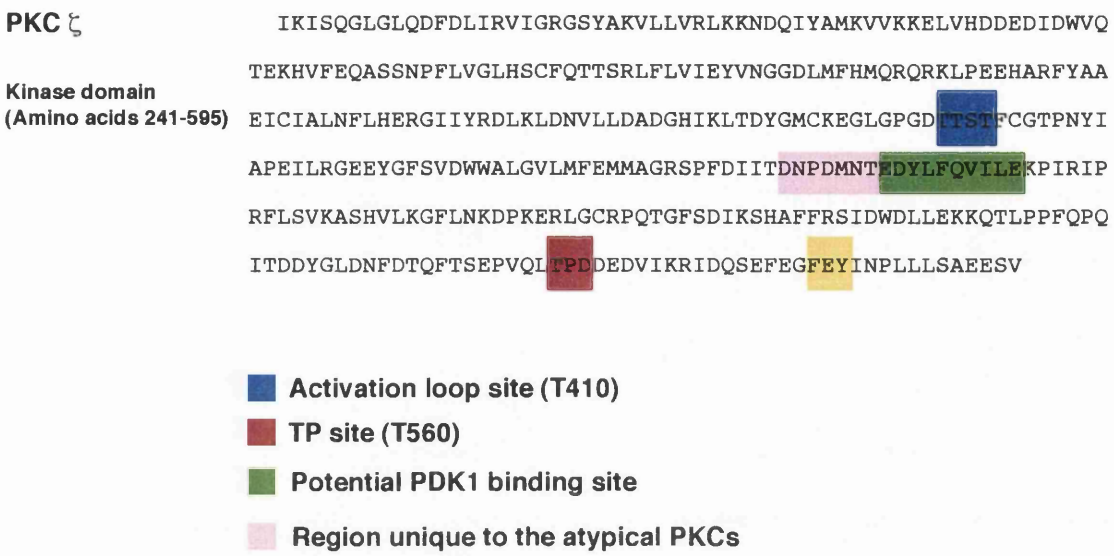


Fig 5.9
The Hypothetical Binding Site of PDK1 to PKC ζ

site is elsewhere in the kinase domain. D.Alessi carried out a two-hybrid screen with PDK1 and pulled out a fragment of PRK2, binding occurring in a C-terminal region of PRK2 (personal communication). The equivalent region in PKC ζ is highlighted in Figure 5.9. Potentially, PDK1 may bind PKC ζ in this same region. Interestingly, this region is close to a region which is distinct in PKC ζ in comparison to all other family members. This may imply some unique influence on the site of interaction.

5.5 PDK1 appears to be the Activation loop kinase for the entire PKC Family

Since all the PKCs have similar activation loop sites, hypothetically an activation loop "PKC ζ kinase" would be able to act on the other PKCs also. Physiologically, such a kinase could be regulated differently for the individual PKC isoforms or there may be a sub-family of "PKC kinases" which may recognise specific PKC isoforms, by either recognising the different regulatory domains, which seems unlikely from the PDK1/ PKC ζ IP data, where the kinase domain alone is sufficient for interaction. However, the following experiments were undertaken, initially *in vitro*, to determine whether PDK1 also phosphorylates other PKC members. Bacterially expressed PKC δ (which has low activation loop site occupancy) was incubated with GST-tagged PDK1 (Figure 5.10). PKC δ is not phosphorylated as strongly as PKC ζ, possibly due to the lack of prior phosphorylation in the C-terminal "FSY" site. This lack of occupancy of all three priming site phosphorylations can lead to protein instability. However PDK1 does increase T505 site phosphorylation. PKC δ activity increased two-fold at 0.2 mol/mol but became inactivated on prolonged incubation (observations by Dr W. Ziegler).

As observed for PKC ζ , the other PKCs - classic, novel and the other atypical PKC (PKC ι) - could be co-immunoprecipitated with co-expressed PDK1 (Figure 5.11). PDK1 was not imunoprecipitated in either the vector control or by another kinase, myc tagged Raf. Therefore, PDK1 displays a broad but selective specificity for interaction with PKC isotypes. As was seen for PKC ζ, the classical and novel PKCs (PKC βII and PKC ε being an example of each) bind to the kinase domain of PDK (Figure 5.12).

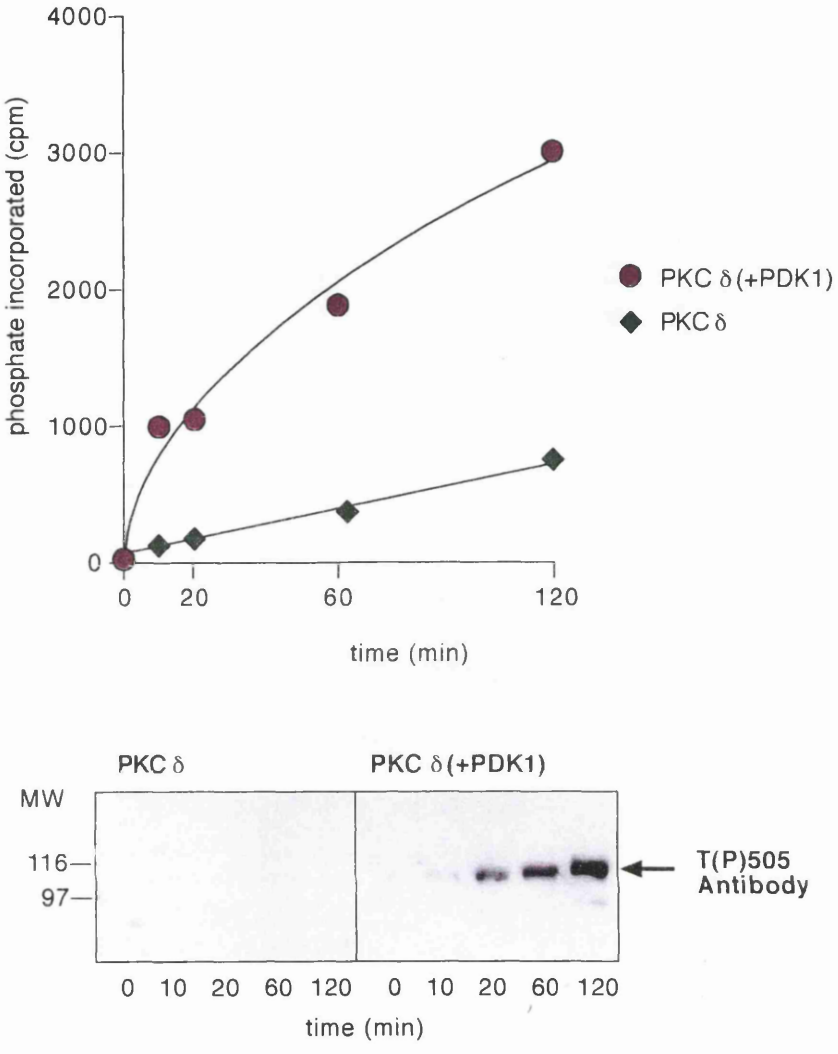


Fig 5.10
***In vitro* Phosphorylation and Activation of PKC δ by PDK1**
Bacterially expressed PKC δ was incubated with GST tagged PDK1. The assay was carried out as in Fig 5.2. Incorporation of ^{32}P phosphate determines the stoichiometry of phosphorylation (Fig 5.10A) and activation loop site phosphorylation (T505) is measured by phospho-specific antibodies (Fig 5.10B).

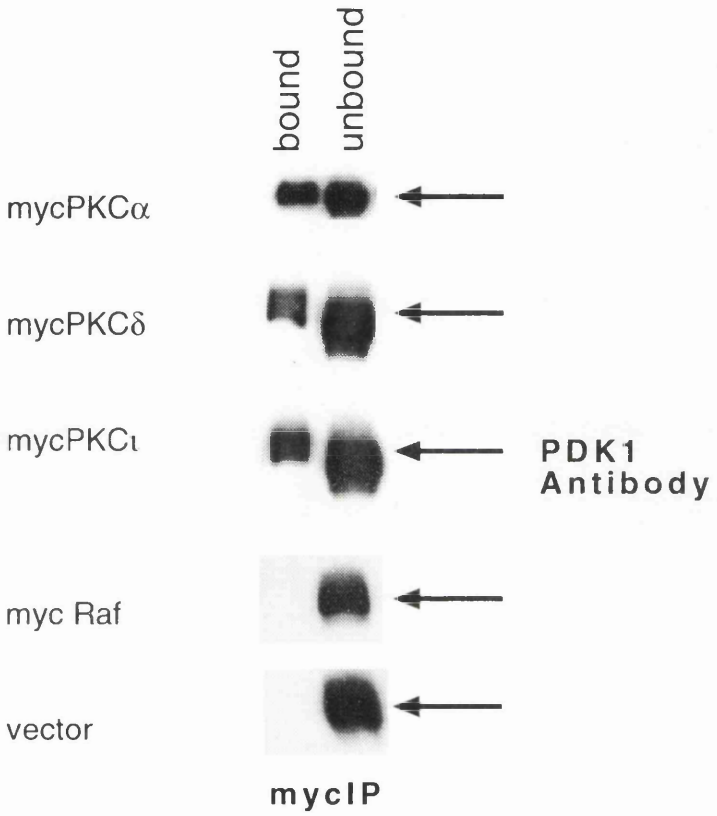


Fig 5.11
PDK1 complexes with classical and novel PKCs
Myc-tagged classical and novel PKCs were expressed with non-tagged PDK1. Any PDK1 bound to PKCs (or Raf) was detected by Western blotting.

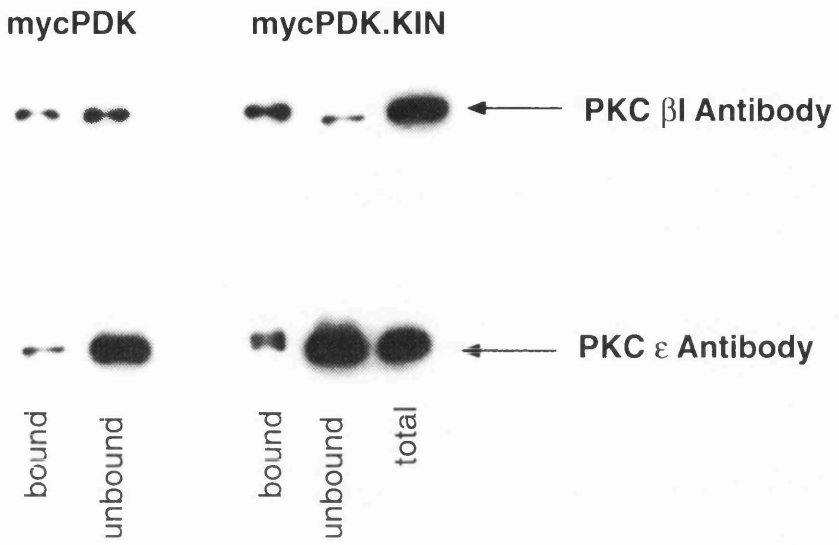


Fig 5.12
The kinase domain of PDK1 complexes with classical and novel PKCs
Classical and novel PKCs, in the form of PKC βI and PKC ε respectively, were expressed with myc-tagged PDK1 full length or kinase domain proteins. Amounts of PKCs which bound to PDK1 were detected by the specific PKC antibodies.

5.6 Regulation of the Activation loop phosphorylation of PKC ζ

PDK1 was originally identified as a PIP_3-dependent kinase activity. Therefore, the next experiments were carried out to identify which lipids are important for the optimal activity of PDK1 and also for PKC. Allosteric effectors, in the form of DAG for c/n PKCs, initiate activation by causing membrane translocation and contribute towards removing the PSS from the catalytic active site. Experiments with PKC δ established that *in vitro* both $PI(3,4,5)P_3$ (for PDK1 activation) and TPA (for PKC δ activation) are required for the highest PKC δ T505 phosphorylation by PDK1 (Figure 5.13, experiments carried out with Dr W. Ziegler).

PDK1 is activated most strongly by $PI(3,4,5)P_3$ (not $PI(3,4)P_2$) (Stephens et al., 1998). Trying to pinpoint which lipids are important for PKC ζ activation was not so straightforward. Previous experiments have established that aPKCs are not sensitive to DAG/TPA (Ways et al., 1992). Several lipids were incubated with PDK1 and PKC ζ to determine what results in maximal T410 phosphorylation of PKC ζ. No lipids tested (ceramide, sphingosine, $PI(4,5)P_2$ or phosphatidic acid) or TPA, gave an increase in PKC ζ activity above that of mixed brain lipids. This suggests that either we have not identified the specific lipid required to maximally activate PKC ζ or the lipid presentation is not of the correct conformation for recognition by PKC ζ. Alternatively, PKC ζ may have a different mechanism of "opening its structure" and exposing the activation loop site. Protein-protein interaction may be required to bind to the PSS region (for example, ZIP/p62 (Puls et al., 1997; Sanchez et al., 1998) or several proteins have been identified which bind specifically to the aPKC zinc fingers), resulting in release from autoinhibition and subsequent activation.

As has been shown previously, PDK1 is activated by biologically relevant stereoisomers of PIP_3 thus the upstream activator of PDK1 must be PI3K. Therefore, *in vivo* it would be expected that the PI3K inhibitor, LY 294002 (Vlahos et al., 1994), would inhibit the action of PDK1. To test this hypothesis, PDK1 and PKC ζ were co-expressed in 293 cells and the cells serum starved for 24h before the LY compound was added (10μM for 1h). Co-expression of PDK1 and PKC ζ increases T410 phosphorylation in comparison to when PKC ζ is expressed alone (Figure 5.14A). This activation loop phosphorylation is inhibited by the LY compound. Moreover,

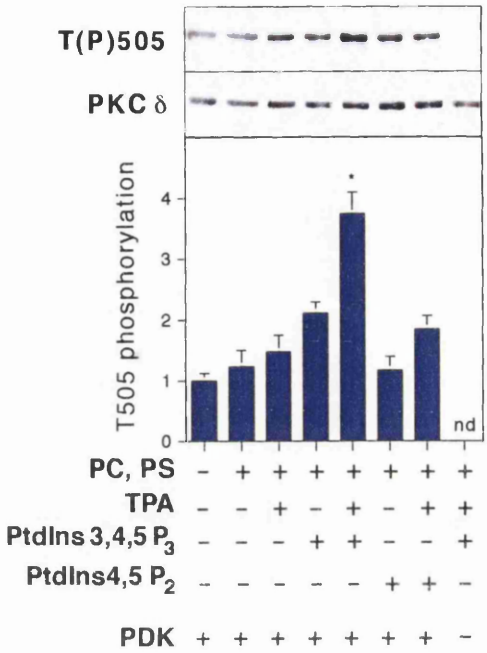


Fig 5.13
Lipids mediate PKC δ Phosphorylation by PDK1
The *in vitro* assay was carried out as previously described for Fig 5.2. PKC δ and PDK were incubated together in the presence of various lipids and TPA. The $PI(4,5)P_2$ concentration used was 10μM and TPA 0.5μM. Levels of T505 phosphorylation were measured using phospho-specific antibodies (*P<0.05, from three experiments).

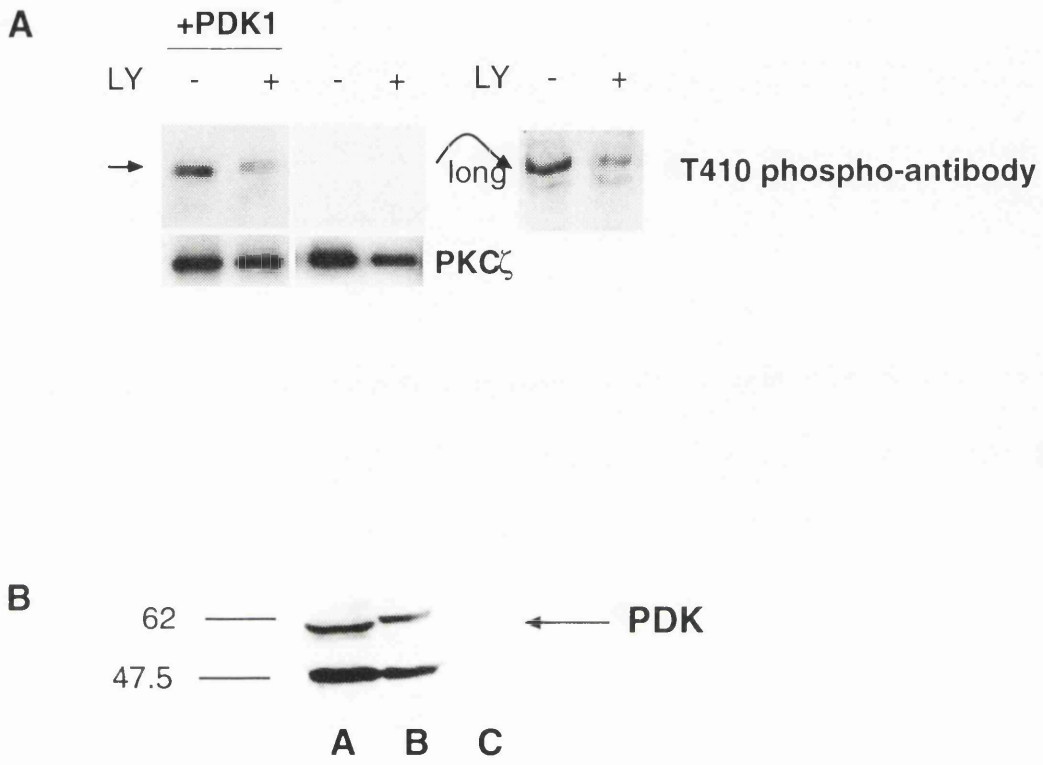


Fig 5.14
LY 294002 inhibits PDK1 induced and endogenous PKC ζ T410 site Phosphorylation
Fig 5.14A shows a Western for T410 occupation in PKC ζ transfected cells, with and without PDK1 as indicated. LY 294002 (LY) was added to cells for 1h prior to harvest. A long exposure (right panel) was required to detect activation loop phosphorylation in the absence of PDK1.

The data was quantified from scanned images and LY treatment inhibited PDK1 phosphorylation of PKCζ by 47% and by 53% for the endogenous T410 kinase.

Cell extracts were blotted for endogenous PDK1 (Fig 5.14B). Lanes:- A. 293 cells, B. COS cells, C. Swiss 3T3 cells.

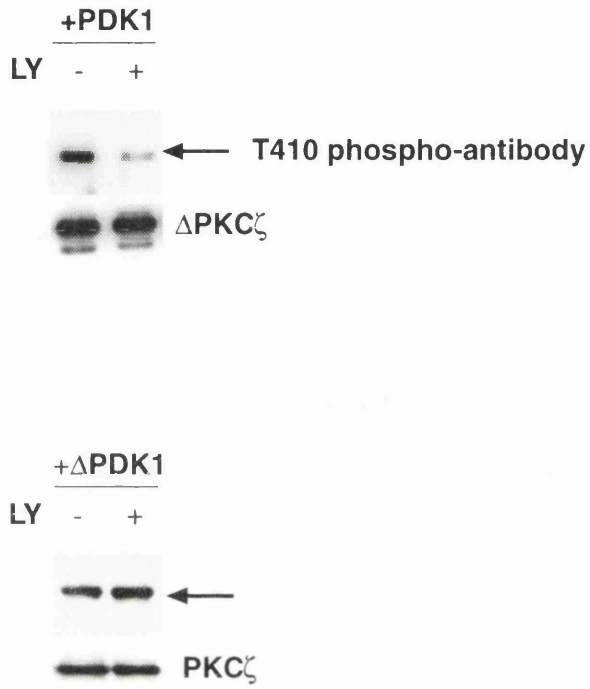


Fig 5.15
Dependence on PI3K of PDK1 Phosphorylation of PKC ζ
PKC ζ kinase domain (Δ PKC ζ) was expressed with full length PDK and PDK kinase domain and PKC ζ were co-expressed in 293 cells. Effects of LY 294002 on the activation loop phosphorylation were monitored. The data was quantified and LY 294002 treatment of PKC ζ kinase (expressed with PDK1) inhibited T410 phosphorylation by 65%.

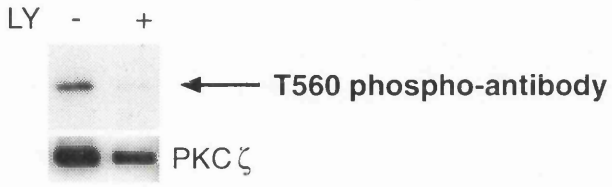


Fig 5.16
PDK1 affects PKC ζ T560 site Phosphorylation
PKC ζ was co-expressed with PDK1. Total lysates were analysed by SDS-PAGE. The effects of LY treatment were determined on T560 site phosphorylation using phospho-specific antibodies.

T410 phosphorylation is decreased by incubation with LY 294002, even if PDK1 is not overexpressed with PKC ζ (Figure 5.14A - on a long exposure). Therefore, an endogenous activation loop kinase is being inhibited. Endogenous PDK1 is present in 293 cells, as well as in COS7 cells but could not be detected in Swiss 3T3 cells (Figure 5.14B).

There appears to be a PI3K-dependent step in the PDK1-induced phosphorylation of PKC ζ. PDK1 is itself not inhibited by any PI3K inhibitors, however lack of PIP_3 production is obviously inhibitory. Therefore to measure whether the PI3K was directed at PDK1, PKC or both, LY294002 sensitivity was monitored in experiments where kinase domain constructs of both were used. In the case of co-expression of PDK1 and PKC ζ-kinase domain (lack of the regulatory domain implies a lack of lipid binding sites), phosphorylation of the T410 site increased and as observed for wild-type PKC ζ, this response was sensitive LY294002 (Figure 5.15A). However, PDK1 kinase domain expression induced wild-type PKC ζ T 410 phosphorylation which was independent of the presence or absence of LY294002 (Figure 5.15B). This is consistent with a model in which the PI3K step operates through the PDK1 regulatory (PH) domain.

As the catalytic activity of PKC ζ increases when T410 phosphorylation increases, it might be expected that a concomitant increase in autophosphorylation occurs. T560 site phosphorylation was determined on co-expression of PKC ζ and PDK1. When activation loop phosphorylation is increased, the TP site is also occupied (Figure 5.16). This phosphorylation is inhibited by LY294002 which mirrors occupation of the activation loop site. However, since this site does not increase substantially on OA treatment, see Chapter 4, phosphorylation of this site may itself be affected by LY treatment.

5.7 Discussion

The substrate specificity of PDK1 is expanding rapidly, however, not surprisingly, it has been limited to the AGC kinase family members. PKB was the first identified substrate and due to having the same consensus sequence, **TXCGTX(E/D)YXAPE**, $p70^{S6K}$ (Alessi et al., 1998; Pullen et al., 1998), PKC (Chou et al., 1998; Dutil et al., 1998; Le Good et al., 1998), PKA (Cheng et al., 1998) and PKB β and γ isoforms (Walker et al., 1998) were also found to be also phosphorylated by PDK1. PKC ζ and PDK1 form a kinase-kinase domain complex and subsequent increases in T410 phosphorylation and PKC ζ activation were observed. In the literature, the aPKCs have been

found to be regulated by $PI(3,4,5)P_3$ (Akimoto et al., 1996) but in the case of PKC ζ, this issue has been somewhat disputed *in vitro* (Nakanishi et al., 1993; Palmer et al., 1995). The regulation by lipids may be explained by the source of PKC ζ. When it is purified from a tissue source, complexed PDK1 may also be isolated and may make PKC ζ appear to be stimulated by $PI(3,4,5)P_3$. In comparison, *in vitro* experiments using baculovirus PKC ζ do not show a marked $PI(3,4,5)P_3$-dependent activation. Data in this chapter demonstrates that PKC ζ T410 site phosphorylation is clearly sensitive to the PI3K inhibitor, LY 294002. This was found to be directed at the PH domain of PDK1 since expression of the PKC ζ kinase domain and PDK1 was still LY sensitive. Therefore, it can be concluded that the role of $PI(3,4,5)P_3$ in PKC ζ activation is channelled *in vivo* through PDK1.

$PI(3,4,5)P_3$ recruitment of PDK1 to the membrane has been shown in porcine aortic endothelial (PAE) cells (Anderson et al., 1998; Currie et al., 1999). Under non-stimulated conditions, PDK1 has a diffuse cytoplasmic staining. On PDGF stimulation, PDK1 is localised on the cell surface, i.e. on the plasma membrane. This translocation is inhibited by wortmannin and is independent of kinase activity of PDK1 (since a kinase dead mutant still translocated to the membrane on PDGF stimulation). If constitutively active PI3K (*p110 PI3K) is co-expressed with PDK1, PDK1 is localised on the plasma membrane. Moreover, if the PH domain is disrupted (R474A) the mutant is less active than the wild-type protein and on PDGF activation, PDK1 does not undergo translocation but cell ruffling still occurs, indicating responsiveness to PI3K activated pathways. PH domains have already been implicated in recruitment of signalling molecules to the cell surface (Lemmon and Ferguson, 1998; Lemmon et al., 1996; Lemmon and Schlessinger, 1998). As well as membrane recruitment, $PI(3,4,5)P_3$ may play a role in removing steric constraints in PDK1 and causing conformational changes. A Δ PH mutant of PKB has increased activity in comparison to the wild-type protein (Kohn et al., 1996), suggesting that the PH domain may sterically block access to the catalytic site (interestingly, PKC ζ has been proposed as binding to the PH domain of PKB (Konishi et al., 1994; Konishi et al., 1994), but how this affects PKB activity is not known). However, the PH domain of PDK1 may affect activity in an analogous manner.

It will be interesting to determine which extracellular stimuli activate PDK1. At present, PDK1 appears to be constitutively active *in vitro* (Alessi et al., 1997). PDK1 is phosphorylated on four Ser residues in unstimulated cells (including S 241, which may be analogous to threonine residues in activation loops)

and phosphorylation is not increased on IGF-1 stimulation. However, no data demonstrates levels of PDK1 activity or phosphorylation on membrane localisation (myristolation constructs) or PDGF stimulation. Why two potentially $PI(3,4,5)P_3$-inducing effectors, PDGF and insulin, affect PDK1 activity differently is still unclear. Insulin stimulation of the insulin receptor substrate, IRS-1, can result in translocation to other membrane compartments, for example, the endosomal membrane. Thus, PDK1 may not be in the correct cellular location for this stimulation (no immunofluoresence data was presented under these conditions) or substrate accessibility.

PDK1 phosphorylation of PKC δ appears to be substrate directed. Only when the allosteric regulators of PKC δ are present (DAG or TPA) is the T505 phosphorylation optimal (interestingly, this is disputed for the cPKCs (Dutil et al., 1998). Since the allosteric activators of PKC ζ are still unknown, this hypothesis cannot yet be confirmed for PKC ζ. The experiments carried out with PKA (the catalytic subunit only) to date demonstrate that PDK1 phosphorylates the T197 site *in vitro. In vivo*, experiments could readily establish if the cAMP induced release of the regulatory domain autoinhibition is sufficient for T197 phosphorylation by PDK1 or if this is also a $PI(3,4,5)P_3$ dependent reaction. It would also be interesting to see if PDK1 can bind PKA and if this results in removal of the regulatory domain from the active site. It would be intriguing to know the role AKAPs might play in this reaction.

For $p70^{S6K}$, activation by PDK1 is a more complex matter. $p70^{S6K}$ requires phosphorylation of several sites before the activation loop site, T229 can be phosphorylated (Pullen and Thomas, 1997). T229 phosphorylation and activation by PDK1 was highest when $p70^{S6K}$ had been disinhibited by either truncation of the autoinhibitory C-terminal tail (Alessi et al., 1998) or mutation of all Pro-directed phosphorylation sites in the C-terminal tail to acidic residues or the T389 to glutamic acid residues (Pullen et al., 1998). Unlike for PKC ζ T410 site phosphorylation, T229 site phosphorylation is not sensitive to PI3K inhibitors, e.g. wortmannin. However, *in vivo*, expression of a kinase dead PDK1 mutant prevented insulin from activating $p70^{S6K}$. Therefore, clearly there are several different problems and the regulation of $p70^{S6K}$ is not straightforward. For maximal activity, T389 phosphorylation must occur prior to T229 phosphorylation, which is a $PI(3,4,5)P_3$-dependent event. However, even if the subsequent activation loop site phosphorylation by PDK1 is activated in a PI3K-dependent manner, structural constraints and self-inhibition of $p70^{S6K}$ may affect activation and mask interpretation of how phosphorylation is regulated. $p70^{S6K}$ may not require $PI(3,4,5)P_3$-mediated

membrane localisation for activation. However, it has been suggested that $p70^{S6K}$ is recruited to the membrane through its interaction with Rac1 or cdc42 (Chou and Blenis, 1996), whether this increases T229 phosphorylation is unclear. Interestingly, PDK1 interaction with PRK is Rho-dependent (P.Flynn, unpublished data). $p70^{S6K}$ may also require prior protein interaction and membrane targetting before it is accessible to PDK1. For $p70^{S6K}$ activation, since it requires sequential inputs and is regulated at several different stages, it may not be the simplest case to solve in terms of PDK1 regulation.

What model can be envisaged for PDK1 activation of the aPKCs? PKC ζ, in unstimulated (but serum maintained) cells, has some activity and can complex with PDK1. It is possible that this binding (which is hypothesised to occur near the end of the kinase domain) overcomes autoinhibition by the PSS, circumventing the requirement for membrane activation. Membrane translocation has not been seen consistently for PKC ζ (Dang et al., 1994; Tippmer et al., 1994), however, membrane targeted PKC ζ (by myristolation) in serum starved cells has been shown to increase T410 site phosphorylation (Chou et al., 1998). This would suggest that membrane recruitment is required for increased activation of PKC ζ and that this reflects co-recruitment of PDK1 in a PI3K-dependent manner. PDK1 binds PKC ζ in the cytosol and it is likely that subsequent membrane targeting is PDK1-$PI(3,4,5)P_3$ directed (see Figure 5.17). What factors are important for the dissociation of the complex remains to be determined. Clearly mutation of the T560 site to a glutamic acid is not sufficient and it appears that the phosphorylation state of PKC ζ does not initiate dissociation.

The role PDK1 plays in the phosphorylation of the other PKCs is probably slightly different. Classical and novel PKCs are localised to the membrane in a well-defined DAG-dependent manner. DAG or other PKC allosteric effectors ensure a more readily accessible activation loop and subsequent $PI(3,4,5)P_3$-mediated interaction via PDK1 may help stabilise membrane localisation and enable activation loop phosphorylation. The role of PDK1 plays in interacting with cPKCs may be as that of a scaffold protein to stabilise inactive cPKCs prior to phosphorylation. It will be interesting to see if phosphorylation can still occur if the PDK1 binding site in PKC is deleted.

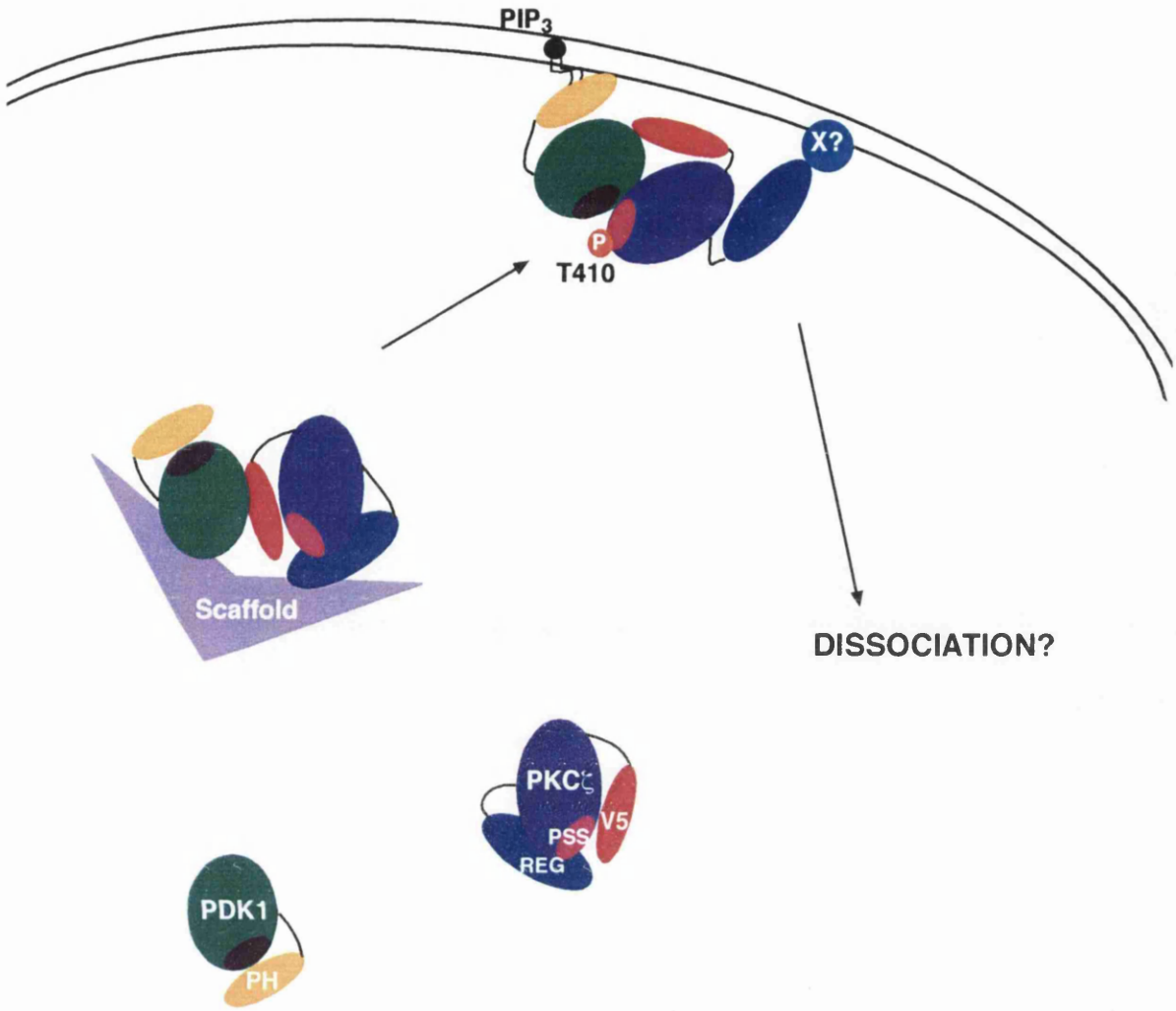


Fig 5.17
PDK1-mediated Activation of PKC ζ

Since there are so many inputs into the PKB, PKC and $p70^{S6K}$ pathways, the answers may come from more detailed analysis of PDK1 itself. If a different readout for PDK1 activity, independent of substrate phosphorylation can be discovered, substrate-kinase regulation scenarios can be unravelled. However, from the results demonstrated here for PKC δ, phosphorylation of the activation loop site lies in the requirements of both the kinase and substrate and correct cellular localisation of both.

Chapter 6

6.1 Introduction

Why has the PKC superfamily become so diverse? What determines PKC isozyme specificity? These are questions which are difficult to answer. Between all the isotypes (especially between classical and novel PKCs) there is no great distinction of cofactor dependencies. The atypical PKCs differ from the other PKCs in that they are not regulated by DAG. The PKCs do not show any particular *in vitro* substrate specificity, however, *in vivo*, individual PKCs do mediate specific and different cellular functions and cause varying responses. Therefore, the preferential targeting of different PKCs to certain subcellular compartments (Goodnight et al., 1995), either as a result of stimulus induced translocation or by binding to intracellular anchoring, scaffold or chaperone proteins, and differential isozyme localisation is probably the key to determining the *in vivo* substrate specificities of the isoforms. For example, PKC α is found in focal contacts bound to several cytoskeletal components, i.e. vinculin and talin (Hyatt et al., 1990; Hyatt et al., 1994).

The literature concerning PKC ζ localisation is extensive. PKC ζ is localised in different cellular compartments dependent on cellular stimuli. For example, PKC ζ is reported to be primarily cytosolic in unstimulated astrocytes and yet is translocated to the membrane on addition of ceramide or sphingomyelinase (Galve-Roperh et al., 1997). Nuclear translocation of PKC ζ occurs during ischemia in rat hearts and is blocked on incubation with wortmannin (Mizukami et al., 1997). In a rat pheochromocytoma cell line, PC12 cells under nerve growth factor (NGF) stimulation, PKC ζ is found to be localised to the nuclear matrix (Wooten et al., 1997). Moreover, stimulation of adipocyte differentiation with insulin or mitogens stimulates nuclear localisation (La Casa et al., 1995), indicating that movement of PKC ζ to the nucleus is not restricted to a specific growth factor. Indeed, nuclear localisation of PKC ζ has been linked to cell cycle regulation. PKC ζ was located in the nucleus in G2 arrested U937 cells (Kiley and Parker, 1995). This was also the case in Swiss 3T3 fibroblasts where transient nuclear PKC ζ localisation was observed as the fibroblasts transited from G2 into mitosis (Kiley and Parker, 1995).

Several other PKCs have been documented as being localised to the nucleus in a stimulus-dependent manner. The nucleus offers a wide range of potential PKC substrates (Chuang et al., 1987; Samuels et al., 1989). For example, laminin B, a nuclear envelope protein, is well documented as being regulated by PKC βII phosphorylation during mitotic lamina disassembly (Hocevar et al., 1993; Hocevar and Fields, 1991). The nucleus contains several of the components required for PKC activation, for example, nuclear phosphoinositidase C β (Divecha et al., 1993; Martelli et al., 1992; Payrastre et al., 1992) and several phospholipids are present in the membrane (Cocco et al., 1989) and calcium is regulated by nuclear $InsP_3$ channels (Stehno et al., 1995; Stehno et al., 1995). Nuclear DAG is formed on phosphatidylcholine breakdown and nuclear DAG levels have been found to increase on cellular stimulation (Banfic et al., 1993; Buchner, 1995). Recently, phosphatidylglycerol, a component of the nuclear membrane, previously isolated as the nuclear membrane activation factor (NMAF, (Murray et al., 1994), was found to activate nuclear PKC βII (Murray and Fields, 1998).

How PKC enters the nucleus has been a topic of much debate. Whether PKC has a nuclear localisation signal (NLS) is unclear. Intracellular macromolecular transport into the nucleus occurs via the nuclear pore complex present in the nuclear membrane (Pante and Aebi, 1996). This occurs in several different ways:- small proteins (40-60kD) or ions and metabolites can freely diffuse through the aqueous diffusion channel in a non-selective manner into the nucleus. However, larger proteins require active import, which is an energy-dependent process. The first step in the uptake process is now characterised as involving a classical NLS. These have been classified in several proteins (Dingwall and Laskey, 1991) but was first identified in the simian virus 40 (SV40) large T antigen (PKKKRKV (Garcia-Bustos et al., 1991). The second class of NLS are basic bipartite repeats, found in nucleoplasmin (KRPAAIKKAGQAKKKK (Gorlich et al., 1996). Ankyrin repeats have also been found to act as NLS (Sachdev et al., 1998). The NLS is recognised in the cytoplasm by importin α or karyopherin α (Gorlich et al., 1994; Moroianu et al., 1995; Moroianu et al., 1995) which interacts with importin β (Gorlich et al., 1996). This complex can then dock onto the nuclear pore complex (NPC) where Ran/TC4 (a Ras-related nuclear GTPase, (Melchior et al., 1993; Moore, 1998; Moore and Blobel, 1993) and NTF2/p10/pp15 help translocate the NLS protein complex in a GTP-dependent manner into the nucleus. Thus protein import is a two-step process. Initial energy-independent substrate binding to the NPC is followed by an energy-dependent translocation, which is independent of the

cytoskeleton. Once inside the nucleus, the complex is dissociated by the GTP form of Ran binding to importin, resulting in release of the transported protein (see (Nigg, 1997) for a detailed review). Several RNAs, including viral genomic RNAs and U snRNAs use the same pathway for nuclear import (Hamm et al., 1990; O'Neill et al., 1995). A second pathway for import of proteins has been identified. A novel NLS signal was found in a heterogeneous nuclear RNA-binding protein, hn RNP A1. hnRNP A1 was found to shuttle between the nucleus and cytoplasm and the sequence important in both import and export was found to be a 38 amino acid long sequence termed M9 (Siomi and Dreyfuss, 1995). M9 is recognised by an M9 specific receptor, transporin which is distantly related to importin β (Nakielny and Dreyfuss, 1996; Nakielny and Dreyfuss, 1998). However, this is not the end of the story. Variations on the classical NLS-dependent protein import have been seen. Import pathways which depend on calcium, calmodulin and ATP (Sweitzer and Hanover, 1996) or are independent of GTP hydrolysis and cytosolic factors (Michael et al., 1997), where a novel hnRNP K nuclear shuttling domain is required, have been described.

It was originally hypothesised that the basic amino acid sequences in the zinc fingers of PKC acted as a bipartite nuclear targeting motif (Malviya and Block, 1992) or that the basic residues in the PSS site were responsible for nuclear translocation. However, this has not been substantiated by any evidence and in fact deletion analysis of PKC α demonstrates that sequences within the hinge region and a C-terminal region are essential for nuclear targeting (James and Olson, 1992). Whether this implies that PKC has an intrinsic NLS signal which is more exposed on activation is unclear. PKC βII appears to be activated within the nucleus during G2 (Goss et al., 1994; Thompson and Fields, 1996), but this does not rule out the presence of a NLS.

Therefore the unique regions required for nuclear localisation of the PKCs have not been isolated. Little data has been published for the details of aPKC nuclear translocation or roles. It may be that localisation is mediated by specific domains or binding proteins. These issues are addressed in this final chapter, where PKC ζ localisation was studied and attempts were made to isolate novel binding proteins.

6.2 Cellular Localisation of PKC ζ

To attempt to identify what determines PKC ζ entry into the nucleus, whether it uses an endogenous NLS or enters bound to another protein, various myc-

tagged domain constructs of PKC ζ were cloned. All the constructs expressed well (Figure 6.1) and thus could be used in immunofluorescence studies.

Wild-type PKC ζ is localised mainly in the cytoplasm (Figure 6.2A). Occasionally, a small amount appears to be in the membrane but cells look healthy and have smooth outer membranes. GFP-PKC ζ (Figure 6.2B) constructs were also expressed (the full length construct was received from Dr Seechiero). These constructs show the same localisation which demonstrates that no differences arise due to the extra 25kD of the GFP protein being bound to PKC ζ. This suggests that wild-type PKC ζ in 10% serum maintained 293 cells does not enter the nucleus. Activation may be required for exposure of a putative NLS, as is seen for NF-κB, where the N terminal domain of IκBα masks the NLS of the homodimer and activation is required for nuclear localisation (Latimer et al., 1998). However, the constitutively active mutants of PKC ζ (Figure 6.2C) are localised in the cytosol. The membrane edges look slightly less smooth, more "active" and ruffled. Without the constraint of the pseudosubstrate autoinhibition, no different localisation was observed. Therefore activation of PKC ζ by pseudosubstrate site "removal" is not sufficient to localise PKC ζ in the nucleus. This is also seen for the phosphorylation site activated mutant (EE), see chapter 4. The kinase domain is mainly cytoplasmic and occasional nuclear and membrane localisation was observed (Figure 6.2D). This suggests that under certain conditions, for example, differences in the cell cycle of individual cells, localisation differs. Cells expressing the kinase domain are more ruffled and cells lift off much more readily from coverslips. These results implicate activated PKC ζ in cytoskeletal architecture.

Expression of the regulatory and smaller Vo domains (Figure 6.3A,B) demonstrated that this domain of PKC ζ could be found in the nucleus. For the smaller constructs, cytoplasmic staining was also visible. It is likely that the smaller proteins (< 45 kD) diffuse into the nucleus, however, the observation that the protein remains there and does not become evenly distributed between the nucleus and cytoplasm suggests that PKC ζ Vo binds to a substrate or targeting protein in the nucleus. Using a GFP-PKC ζ regulatory domain construct (of molecular weight 60kD), PKC ζ was still localised strongly in the nucleus (Figure 6.3C). The co-localisation of the PKC ζ regulatory domain with DAPI confirmed nuclear localisation (Figure 6.3D). The staining was diffuse and not specifically localised in either the nucleolus or nuclear matrix. The PSS sequence is not sufficient for nuclear localisation since deletion of this region still resulted in nuclear localisation of the

regulatory domain (Figure 6.4A). However, this does not rule out diffusion. Binuclear cells were seen in 10% of cells transfected with the regulatory domain construct. It is not clear if this is due to inhibition of cytokinesis. Expression of the regulatory domain of PKC ζ and wild-type protein together did not affect the localisation of either protein. This demonstrates that PKC ζ does not homodimerise (by regulatory domain interaction) and prevent or promote nuclear localisation.

Since the regulatory domain alone is capable of entering the nucleus, the following experiments were carried out to determine under which conditions full length PKC ζ enters the nucleus. It its known that PKC translocation is agonist-dependent. The biological activators of aPKCs have not been described, however, a few conditions were tried, ranging from insulin treatment, osmotic shock to TPA. None of the treatments tried resulted in nuclear localisation of PKC ζ. Since nuclear localisation of PKC ζ has been linked to the cell cycle, several different approaches were used to try to arrest cells in G2 or M phase. Two different methods were tried initially, chemically, by treating cells with nocodazole or attempting to synchronise growth by serum starvation of 293 cells. No success was achieved by either of these methods due to cell death. A second approach was used. Mutants of cyclin B2 or a cdc2 deletion mutant were expressed and caused mitotic arrest (Gallant and Nigg, 1992). In arrested cells mitotic spindles were seen (Figure 6.4C). However, in the cell type studied, no nuclear localisation of PKC ζ or concomitant PKC ζ staining in G2 arrested cells was observed (Figure 6.4B). Thus the G2 localisation is not universal. There is also no obvious colocalisation of PKC ζ to the central part of microtubule spindles, contrary to shark epithelial cells (Lehrich and Forrest, 1994).

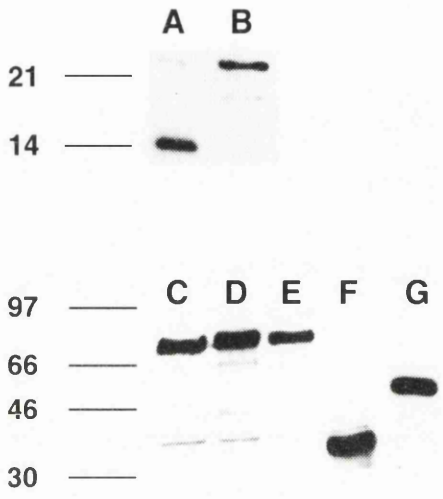


Fig 6.1
Expression of Myc tagged PKC ζ constructs
All PKC ζ constructs were transfected into 293 cells and analysed by SDS-PAGE. Gels were blotted with the myc antibody.
Lanes:- A. Vo, B. Vo+PSS, C. PKC ζ, D. PKC ζ A119E, E. PKC ζ Δ PSS, F. PKC ζ Regulatory domain, G. PKC ζ kinase domain

Fig 6.2

Cellular Localisation of PKC ζ in 293 cells

Cells were transfected with various PKC ζ constructs and localisation analysed by immunofluorescence (see materials and methods). Cells were stained with phalloidin (0.1μg/ml for 15min) to visualise actin and FITC-conjugated antibody to recognise PKC ζ.

Lanes:- A. Myc-PKC ζ, B. GFP-PKC ζ, C. PKC ζ Δ PSS, D. PKC ζ kinase domain

Fig 6.3

Nuclear Localisation of PKC ζ

293 cells were transfected with various PKC ζ regulatory domain constructs and visualised with FITC-conjugated antibody to recognise PKC ζ. Actin was stained with phalloidin, as before. In Fig 6.3D, nuclei were visualised using DAPI.

Lanes:- A. PKC ζ regulatory domain, B. PKC ζ Vo domain, C. GFP-PKC ζ regulatory domain, D. PKC ζ regulatory domain

Fig 6.4

Cellular Localisation of PKC ζ

293 cells were transfected with myc-tagged PKC ζ constructs and stained with FITC-conjugated antibody (Fig 6.4A) or Cy3-conjugated antibody (Fig 6.4B). In Fig 6.4A, cells were transfected with myc-tagged PKC ζ regulatory domain Δ PSS. In Fig 6.4B, 293 cells were cotransfected with PKC ζ and a cyclin B2 mutant. Tubulin was stained directly with a FITC-conjugated antibody. Fig 6.4C demonstrates cell cycle arrest on expression of the cyclin B2 mutant alone.

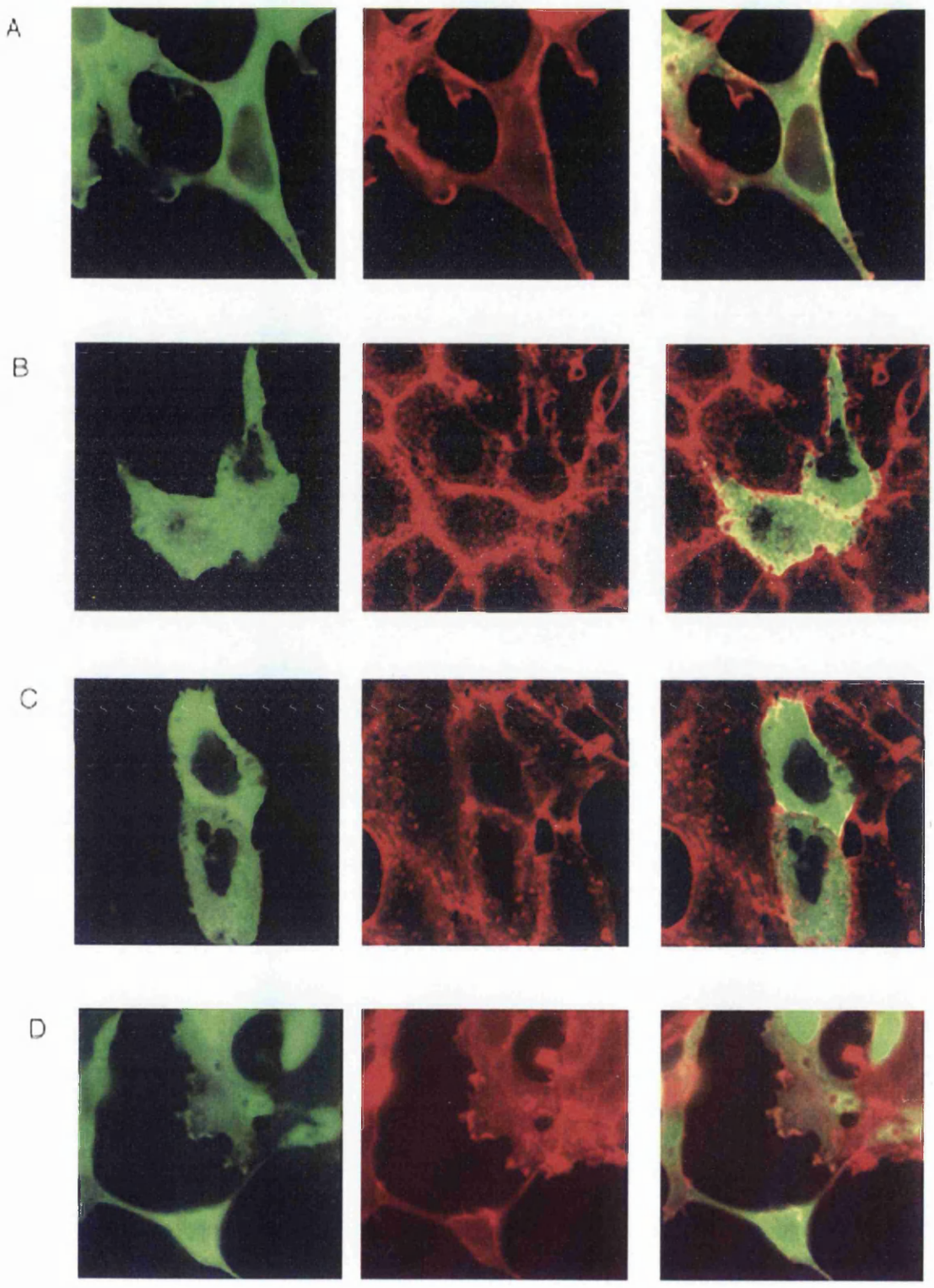


Fig 6.2

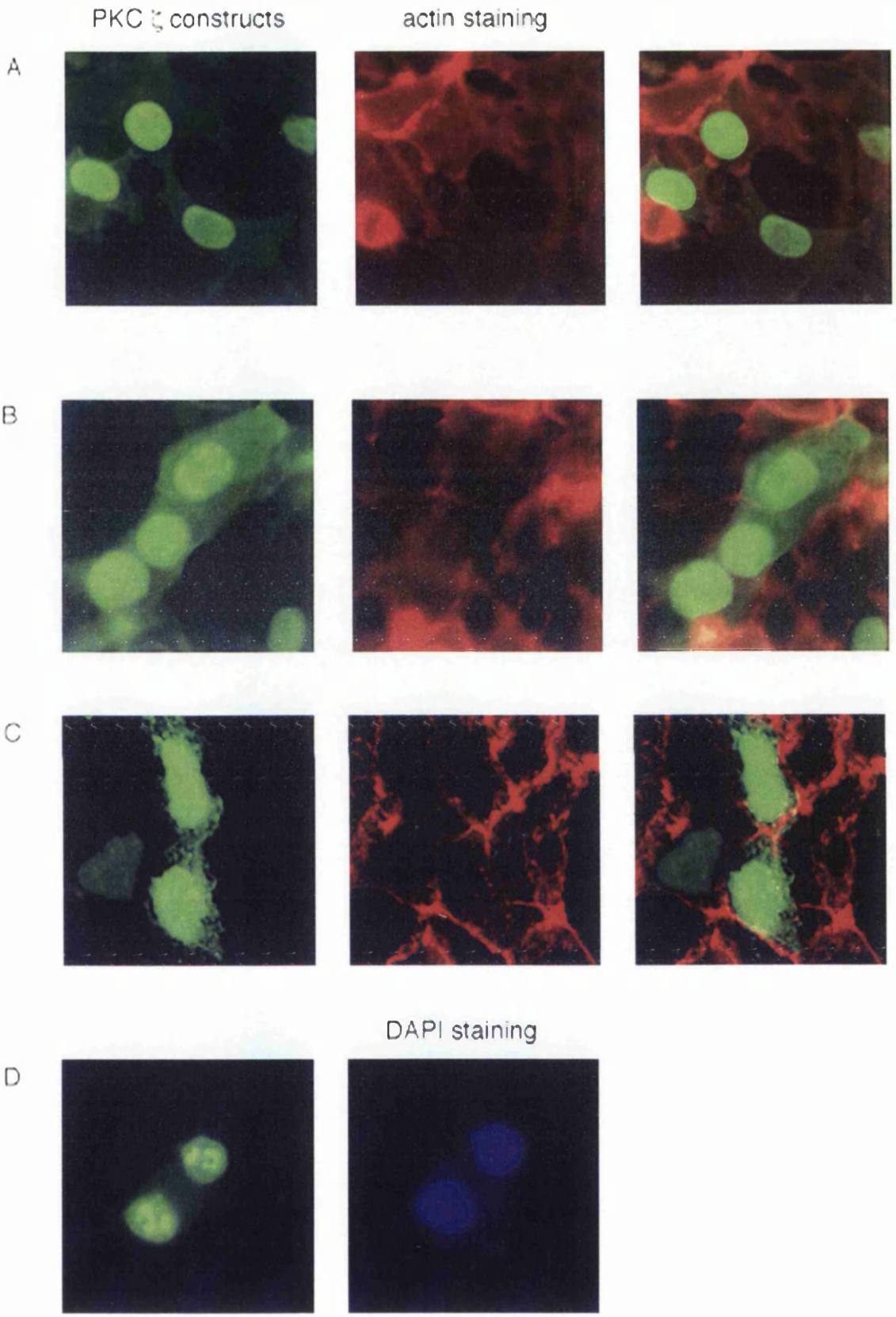
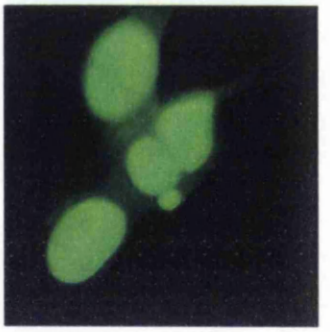


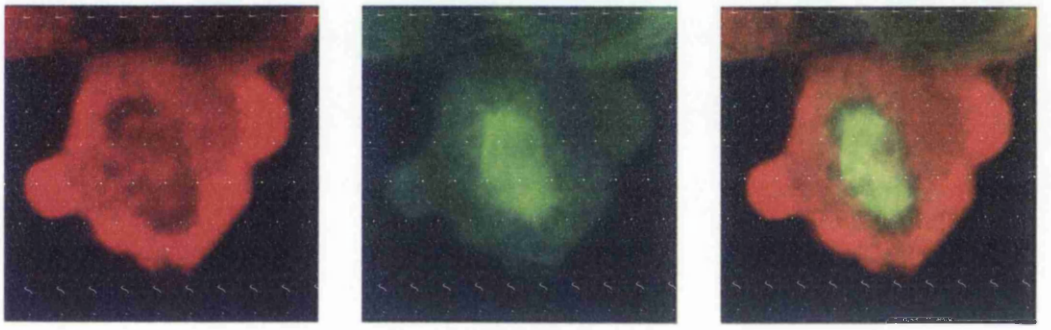
Fig 6.3

A



PKC ζ constructs

B



PKC ζ constructs tubulin staining

C

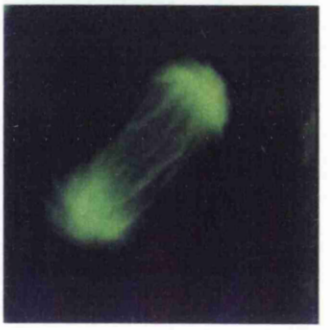


Fig 6.4

6.3 PKC ζ binding proteins

To address why the PKC ζ Vo domain accumulates in the nucleus, the following experiments were carried out to try to isolate novel binding proteins. In particular, to try to isolate any nuclear binding proteins. Several *in vitro* "pulldown" experiments were carried out to identify any nuclear proteins able to bind to the regulatory domain of PKC ζ. For these studies, bacterial proteins were required. Even though HIS tagged proteins expressed well and were detectable by Western blotting (Figure 6.5) hardly any protein was obtained by elution. In comparison, expression of the smaller (GST tagged) domains (Vo or Vo+PSS) was very high (Figure 6.6 -eluted protein). These constructs were used to try to isolate nuclear binding proteins, since it is these which accumulate in the nucleus in preference to the cytosol. The bacterial fusion proteins were purified on beads and then incubated with either cell extracts (at least 1x 10^6 cells) or nuclear extracts (Figure 6.7). No difference in binding proteins were noted between PKC ζ or vector control pulldowns. Thus no obvious binding proteins were isolated, possibly because only soluble nuclear extracts were isolated.

To try to isolate *in vivo* PKC ζ binding proteins, different domain constructs of PKC ζ were expressed in 293 cells and protein labelled with ^{35}S methionine before immunoprecipitation (Figure 6.8). No specific binding proteins were consistently isolated. However, the protein may not be in the correct conformation to bind certain proteins, for example, activation may be required. Interestingly, a protein bound to PKC ζ (Figure 6.9), possibly endogenous PDK1 or a recently identified PKC ζ interacting protein (ZIP (Puls et al., 1997) or p62 (Sanchez et al., 1998)) protein or more likely, a novel binding protein, since it is slightly larger in size (75kD). This protein is not isolated on insulin treatment of cells. It is tempting to speculate that this protein binds and maybe targets inactive PKC ζ to the cytosol and that on insulin stimulation, releases PKC ζ for translocation.

6.4 A PKC ζ nuclear binding protein

During the course of these binding studies, Zhou *et al* showed that nucleolin was a PKC ζ substrate (Zhou et al., 1997). In NGF treated PC12 cells, PKC ζ was localised in the nucleus and phosphorylated a 106 kD nuclear protein. Phosphorylation was blocked by expressing a dominant negative PKC ζ and purified PKC ζ increased p106 phosphorylation from nuclear extracts. The

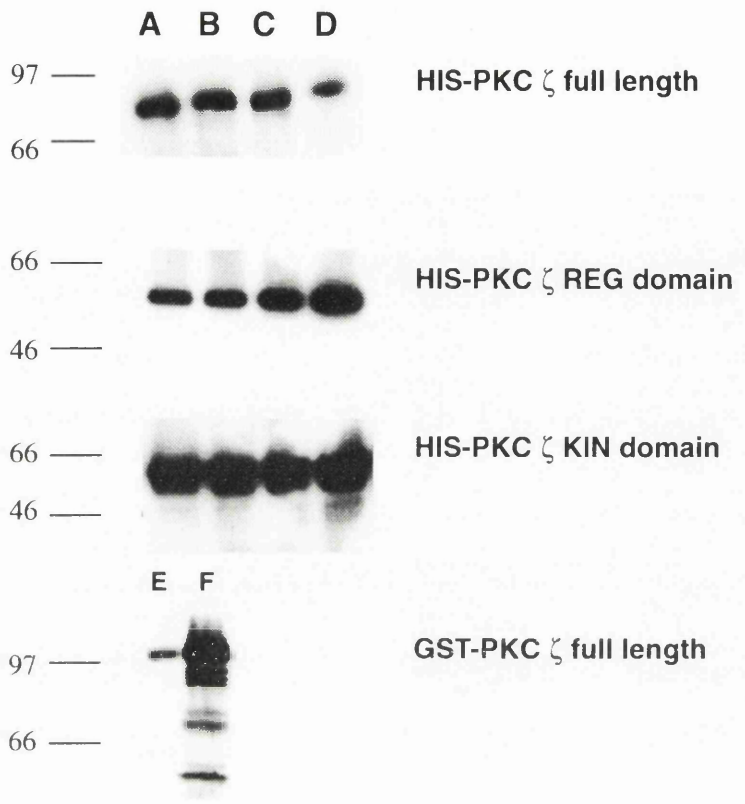


Fig 6.5
Expression of Bacterial PKC ζ
HIS tagged PKC ζ constructs were expressed in BL21 DE3 cells. Protein was detected with PKC ζ antibody.
Lanes:- A. uninduced, B. 1h, C. 2h, D. 3h induction (25°C, 100μM IPTG) GST tagged PKC ζ was also expressed, E. uninduced, F. 3h induction (30°C, 100μM IPTG)

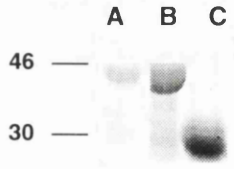


Fig 6.6
Expression of GST- tagged PKC ζ domains
Protein was eluted from GST Sepharose beads with 50mM reduced glutathione.
Lanes:- A. Vo +PSS, B. Vo, C. GST

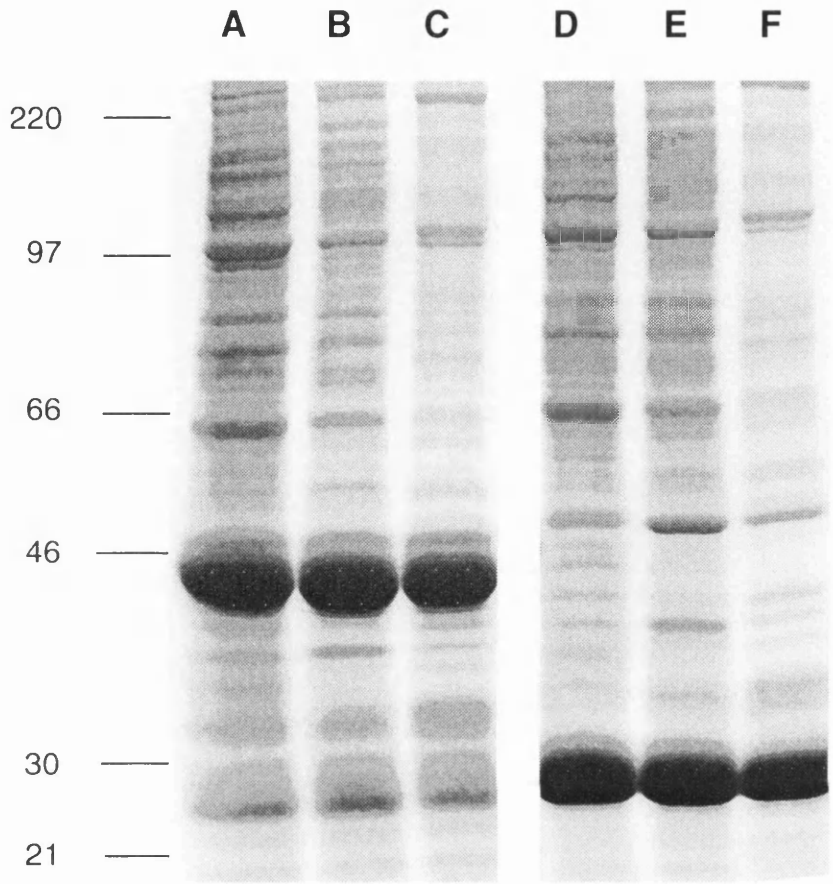


Fig 6.7
PKC ζ fusion protein incubation with nuclear extracts
GST tagged PKC ζ Vo+PSS domain proteins were incubated with soluble nuclear extracts and binding proteins isolated by SDS-PAGE and coomassie staining.
Lanes:- A+D. 293 cells with Vo+PSS and GST control
B+E. COS cells with Vo+PSS and GST
C+F. Swiss 3T3 cells with Vo+PSS and GST

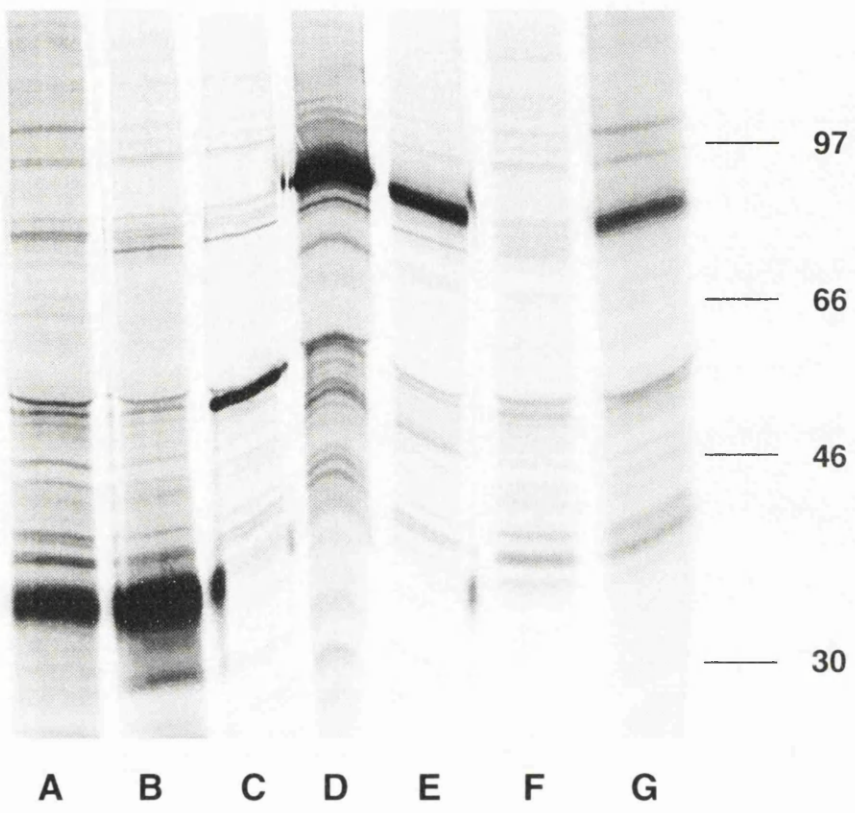


Fig 6.8
PKC ζ *in vivo* binding proteins
293 cells were transfected with various myc-tagged PKC ζ constructs. The cells were starved prior to ^{35}S-methionine labelling and PKC ζ constructs were pulled down by immunoprecipitation and any binding proteins visualised by SDS-PAGE and autoradiography.

The following proteins were immunoprecipitated from 10% FCS maintained cells:- A. regulatory domain, B. regulatory domain Δ PSS, C. kinase domain, D. PKC ζ wild-type, E. PKC ζ A119E, F. vector control, G. PKC ζ Δ PSS

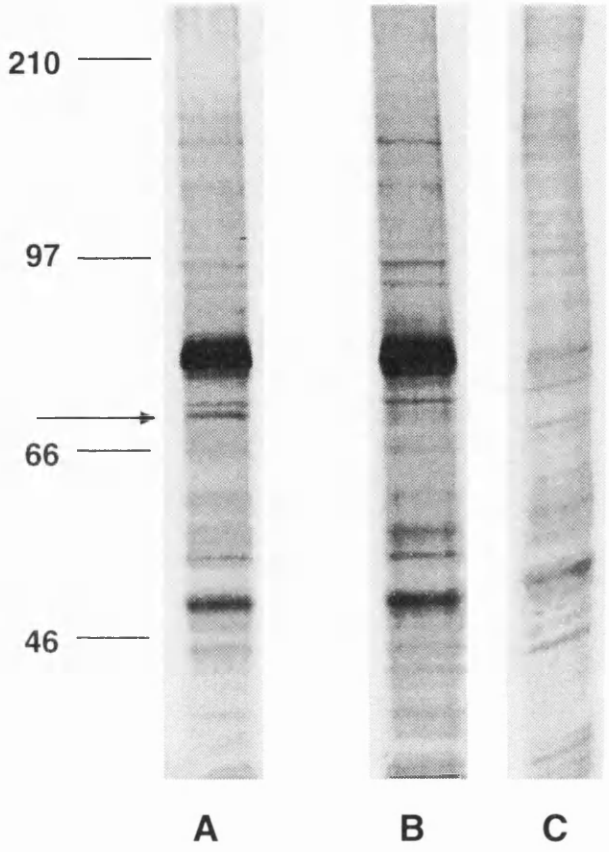


Fig 6.9
PKC ζ binding proteins
To identify if PKC ζ binds to any cellular proteins, cells were stimulated (here, with insulin) before immunoprecipitating PKC ζ. Proteins were labelled with ^{35}S-methionine. Any bound proteins were compared to vector controls, where non-specific binding to Sepharose is seen.The arrow denotes a potential binding protein.

Lanes:- A. PKC ζ, B. PKC ζ +insulin (50μg/ml) for 15min, C. vector

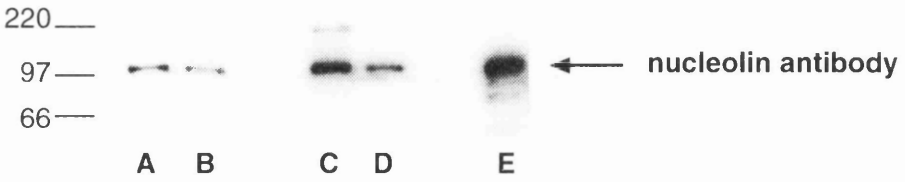


Fig 6.10
***In vitro* interaction of PKC ζ and nucleolin**
Various bacterial GST constructs were incubated with purified nucleolin and any bound nucleolin measured by Western analysis.

Lanes:- A. GST - bound fraction, B. GST - unbound, C. Vo+PSS - bound fraction, D. Vo+PSS - unbound, E. control - 1μg nucleolin

p106 protein was sequenced and identified as nucleolin. Immunofluorescence studies demonstrated that nucleolin and PKC ζ were localised together and accumulated in a perinuclear region. Nucleolin plays a role in the organisation of nucleolar chromatin; packaging of pre-rRNA; rDNA transcription and ribosome assembly. Nucleolin is a substrate for various cell cycle controlled kinases, casein kinase II and cdc 2 kinase. Nucleolin shuttles between the cytoplasm and nucleus. This first identified nuclear *in vivo* PKC ζ substrate, made it tempting to speculate that nucleolin was also a binding protein for PKC ζ.

An *in vitro* binding assay was carried out. GST tagged PKC ζ Vo domain was incubated with purified nucleolin (kindly given by Dr Bouvet) (Figure 6.10). The PKC ζ domain bound to purified nucleolin.

Discussion

In this chapter, the regulatory domain was found to accumulate in the nucleus. The mechanism of entry into the nucleus is unknown. The basic residues in the PSS may comprise a putative NLS, however, in view of the literature and data presented here, this seems unlikely. A regulatory domain construct with the PSS deleted was still found in the nucleus, however, entry driven by diffusion can not be ruled out. Diffusion of small proteins has been reported to be inhibited by treatment with bis (2-aminophenoxy) ethane-N,N,N',N'-tetracetic acid (BAPTA), which depletes nuclear calcium stores. Thus, the use of various chemical inhibitors may be able to determine the mechanism of nuclear import. If BAPTA blocked nuclear localisation of the regulatory domain, this would implicate diffusion as the mechanism of entry. NLS-driven nuclear uptake is inhibited by wheat germ agglutinin (WGA, (Adam et al., 1990) and mAb 414, an antibody to the nuclear pore complex, p62. Interestingly, recent evidence suggests that nuclear uptake of PKC α occurs in a NLS-independent manner and does not require importin β or GTP and uptake is not inhibited by WGA or mAb 414 (Schmalz et al., 1998). Nuclear localisation requires the integrity of the cytoskeleton, thus it may be speculated that a novel PKC import factor is localised and only accessible in an intact cytoskeletal network. However, PKA on cAMP stimulation and release from the regulatory subunit, which tethers the catalytic domain in the cytosol, enters the nucleus by diffusion where it can phosphorylation CREB and other transcription factors. The export of PKA is controlled by binding of PKI to PKA, which exposes the nuclear export sequence (NES) of PKI, resulting in rapid nuclear export (Wen et al., 1995). Activation in the cytosol by

specific agonists, followed by nuclear diffusion and subsequent controlled export may be relevant to the PKC family too.

The lack of finding any conditions which translocated full length PKC ζ into the nucleus made it difficult to analyse sequences important in nuclear uptake. This technical difficulty may be overcome by being able to study "live" cells. It would be very interesting to repeat the studies using GFP-PKC ζ and use time lapse imaging to try to dissect the role of PKC ζ in nuclear function. If a stimulus for entry is identified, then the various mutant constructs can be used to analyse which structural features, for example, which domain or activated form of PKC ζ, is sufficient for import. Activation alone does not appear to be sufficient for entry into the nucleus, however, phosphorylation and lipid activation together (in the form of a PKC ζ Δ PSS/EE mutant) may cause nuclear translocation. Alternatively, PKC ζ may be regulated in a similar manner to PKC βII, where activation of PKC βII in the catalytic domain occurs within the nucleus (Walker et al., 1995). It is interesting to note that insulin or PI3K-dependent pathways have been documented to stimulate nuclear localisation (as has also been seen for PKB (Meier et al., 1997). Whether this implies that activated PKC ζ is required (via PDK1) for nuclear uptake is unclear. However, since the phosphorylation site mutants are not localised in the nucleus, see chapter 4, it may suggest that the mechanism of uptake is sensitive to wortmannin (possibly via vesicle trafficking).

How PKC ζ enters the nucleus is unknown. PKC ζ may translocate to the nucleus bound to cell cycle-dependent kinases, given that PKC ζ can enter the nucleus in a cell cycle dependent manner. Alternatively, different stages in the cell cycle may regulate different phospholipid metabolism (Jackowski, 1996) or calcium levels, which may act to stimulate PKC ζ nuclear uptake directly, or via other proteins. Other nucleocytoplasmic shuttling proteins may take PKC ζ with them to the nucleus, for example the novel binding protein, nucleolin. Further experiments to elucidate the role nucleolin may play in targeting PKC ζ to the nucleus, when PKC ζ is activated to phosphorylate nucleolin and the role of PKC ζ, once it has phosphorylated and dissociated from nucleolin, in the nucleus will be intriguing. Unfortunately, the nucleolin antibody is not good enough to be used in immunofluorescence. It will be intriguing to discover the role of PKC ζ in the nucleus. It has been implicated in mitogenesis but does not appear to modulate nuclear envelope breakdown. PKC ζ may be required for nuclear envelope assembly after mitosis. It will be interesting to discover what controls nuclear export of PKC ζ, since this may give insight into its role.

The expression of several different domain constructs of PKC ζ has enabled the analysis of their cellular localisation and enabled possible identification of specific domain binding proteins. Two different methods of "pull down" experiments were tried, either by immunoprecipitation with ^{35}S labelled proteins or incubation with cell extracts. The principle of these "pull down" experiments is that PKC ζ intrinsically binds other proteins, independent of cell type or cellular stimulation. For example, initially inactive PKC ζ may bind a chaperone protein required to take it to the membrane or PKC ζ may bind a scaffold protein, which may then expose certain domains to other proteins. A potential binding protein was visualised and given more time, such approaches may indeed be able to identify and characterise further novel PKC ζ binding proteins. However, the binding assay may need to be more sensitive. To overcome some technical problems and difficulties associated with overexpression, immunoprecipitation of the endogenous protein and any endogenous binding proteins would be interesting. The PKC ζ antibodies do not appear to be sensitive enough to quantitatively isolate binding proteins. Crosslinking the antibody may help to pull out more binding proteins. However, certain proteins may only bind PKC ζ when it is in its activated either structurally (in the form of the constitutively active constructs) or phosphorylation site mutants or on cellular activation. Interestingly, the recently isolated atypical PKC-isotype specific interacting protein (ASIP) was identified on screening a NIH 3T3 cDNA expression library with an autophosphorylated PKC ζ probe (Izumi et al., 1998). The binding protein itself may also require activation by growth factors or other effectors, for example lipid binding, before being able to recognise PKC ζ.

Alternative technical strategies may have to be employed, for example, a yeast two-hybrid screen was used to independently isolate a PKC ζ binding protein ZIP (from a rat brain library (Puls et al., 1997)) or p62 (from a kidney library (Sanchez et al., 1998)). The role of ZIP/p62 is controversial. One group places it as targeting PKC ζ to the late endosomal pathway, whereas the other group believe PKC ζ may tether ZIP in the cytoplasm. Therefore, the interaction is important in PKC ζ localisation but how is not clear. A method to analyse PKC ζ binding proteins within the cell would be ideal, however, this is only possible (by immunofluorescence or FRET) if the binding protein has already been identified. More in depth characterisation of the role of endogenous PKCs within the cell may provide more answers to its function

and localisation. The identity of physiological binding proteins and the role they play in PKC ζ localisation and function has yet to be determined.

Chapter 7

7.1 The atypical PKC subfamily

The aPKCs comprise an expanding subfamily of PKCs. On the genomic level, there are now several genes, of which two are potentially transcribed pseudogenes, ΨPKC ζ and PKC ζ Ψ I, the pseudogene isolated during the course of this thesis. In light of the lack of overt phenotype of the PKC ζ knockout and given that PKC ζ transcripts are still detected in PKC ζ knockout mice, it seems likely that either of the pseudogenes is transcribed and translated in the knockout and is capable of taking over PKC ζ wild-type function, or there is a redundancy amongst the aPKCs and PKC ι function is dominant in PKC ζ knockout mice. On going collaboration with M.Leitges in PKC ζ knockout mice will hopefully clarify this question.

The expansion of this subfamily in mammalian cells suggests that the aPKCs play important and diverse roles in multicellular organisms. *S.cerevisiae* has a single PKC (Levin et al., 1990; Yoshida et al., 1992) (*S.pombe* has two isoforms (Mazzei et al., 1993), which is a compilation of various c/n/a PKC and PRK domains. A less complicated multicellular organism, *C.elegans* has c/n PKCs (Islas-Trejo et al., 1997; Land et al., 1994; Tabuse et al., 1989) but only one composite aPKC termed PKC3 (which is 50% identical to both PKC ζ and PKC ι (Wu et al., 1998). Being able to study aPKC function in a complete, less complicated system will give valuable insights into the possible co-ordinated role of aPKCs. If the role of the aPKCs can be understood in an entire organism, then discrepancies or apparently conflicting results can be explained in different tissues or cell types. The roles of PKC3 in *C.elegans* reflect functions of both PKC ζ and PKC ι. PKC 3 is crucial for embryogenesis (PKC ι knockout results in embryonic lethality, M.Leitges unpublished data, possibly due to endothelial cell apoptosis, as has been implicated for Raf knockouts (Wojnowski et al., 1997); plays a role in highly differentiated tissues (PKC ζ has been implicated in mitogenesis and differentiation (Wooten et al., 1994), for example in U937 cells (Ways et al., 1994) and preventing apoptosis (PKC ι has been implicated as being a survival factor) and is regulated by physical stresses (both aPKCs are involved in UV-induced pathways (Berra et al., 1997; Huang et al., 1997; Murray and Fields, 1997). Recently, PKC3 has been implicated in establishing cell polarity. Polarity (par) genes are involved in partitioning molecules in the first asymmetric division of the zygote (Guo and Kemphues, 1996). PKC3 was found to associate and interact directly (*in vitro* and *in vivo*)

with PAR3 and reduction of PKC3 levels in embryos (by RNA depletion), caused phenotypic defects consistent with Par3 and Par6 mutants (Bowerman et al., 1997; Tabuse et al., 1998). It appears that PAR 6 complexes with PAR 3 and PKC3 together (Hung and Kemphues, 1998). Interestingly, a mammalian homologue of PAR3 has been identified which interacts with the kinase domain of aPKCs - atypical PKC-isotype specific interacting protein, ASIP (Izumi et al., 1998). ASIP colocalised with PKC λ at cell junctions in epithelial MDCK II cells. Thus the asymmetric distribution and role of aPKCs in affecting cell polarity is clearly conserved throughout evolution. Studying other organisms will give valuble insights into the role of aPKCs.

Clearly, both aPKCs have similar and yet distinct roles. The kinase domains are highly homologous and the greatest differences occur in the regulatory domains (Selbie et al., 1993). The high sequence homology at the C-terminus also makes it difficult to distinguish between the two proteins, however, an amino-terminal PKC ι antibody can resolve various discrepancies. Indeed, in the literature the two have been confused and several observations with *Xenopus* oocyte PKC ζ are in fact giving insight into the role of PKC ι (Dominguez et al., 1992). Most research has been carried out on PKC ζ, possibly due to the more recent identification of PKC ι and PKC λ. Thus it is more difficult to assess if aPKCs play homologous roles in cellular regulation or if experiments were just not carried out to with both isoforms (especially, the effects of EGF/PDGF (Akimoto et al., 1996). Do the two isoforms act co-operatively or antagonistically to each other? Both PKC ζ and PKC ι are ubiquitously expressed. Certain cell types, for example neutrophils, only express one aPKC and thus may be a useful experimental system to study each specific isoform. Why certain aPKCs are not present in certain cell types will be more difficult to analyse but may give great insight into the cellular pathways each can mediate.

7.2 Mechanism of activation of aPKCs

How does the activation of aPKCs differ from the model postulated for PKC α? Mechanistically, since neither DAG nor calcium or indeed evident membrane localisation is required for PKC ζ activation, what is required to remove the autoinhibition imposed by the PSS sequence in the active site? Since the aPKCs already have a negatively charged amino acid in the most C-terminal phosphorylation site, the V5 domain may not be masking the active site, as has been postulated for PKA. Therefore removal of the PSS

site may be less energetically demanding due to the active site being more accessible initially. Lipid mediated stimulation may be required for activation. Ceramides were postulated to bind PKC ζ *in vitro* in a concentration-dependent manner (Lozano et al., 1994; Muller et al., 1995) however, this has been disputed recently (Huwiler et al., 1998). Thus, the effects of ceramides may be via upstream kinases or phosphatases, see chapter 4. Acidic lipids (PA, bisPA) bind to and activate PKC ζ, as seen by electrophoretic shift (Limatola et al., 1994). The mobility shift may potentially be due to increased T560 phosphorylation, not PA binding. We did not see any increase in activation of PKC ζ on prior incubation with PA, when measuring PKC ζ phosphorylation and activation by PDK1, see chapter 5. Thus the direct role of lipids in mediating PKC ζ activity is unclear. Lipid binding to the C1 region may help stabilise protein-protein interaction. The C1 domain of Raf stabilises the interaction with the Ras binding domain by binding to a Ras prenyl group (Luo et al., 1997). Ceramide binding to Raf1 helps mediate this interaction (Muller et al., 1998). This may be true for PKC ζ since ceramide promoted aPKC binding to par 4 (Diaz-Meco et al., 1996).

Alternatively, any zinc finger (par 4 or LIP) or PSS binding protein (p62 or ZIP) may mediate activation. Protein interaction, for example PDK1 binding at a more C-terminal site, may result in conformational changes permitting substrate phosphorylation. The zinc finger domain binding proteins either increase (LIP (Diaz-Meco et al., 1996) or inhibit (par4) aPKC activity. The regulation of aPKCs would then be indirectly controlled by protein interactions to external stimuli, for example PI3K or E-cadherin (Eaton and Simons, 1995). This may explain why lipids alone do not result in large increases in *in vitro* activity assays. It will be interesting to be able to produce enough soluble protein to enable crystallisation of either the kinase domain or full length protein or co-crystallisation of aPKCs with interacting proteins to see how this changes the structural conformation of the aPKCs.

The aPKCs may differ from c/nPKCs in the regulation of phosphorylation. The rate of phosphorylation is certainly more dynamic (chapter 4) and regulation of aPKCs may be more similar to that of PKB. However, it is unclear what role membrane localisation plays in phosphorylation and activation. Membrane localisation may be required for finding substrates.

The ability we have to measure activity of the aPKCs is not reproducible. Why this is different from other PKCs is unclear. The PSS peptide may not be the best substrate and another protein (i.e. in a similar scenario to Raf-MAPK)

needs to be discovered as a substrate to reflect PKC ζ activity. It may be that PKC δ, as presented in chapter 4, may be the *in vivo* substrate and be a potential readout for PKC ζ activity. Alternatively, the correct lipid environment or optimal lipid presentation must be discovered to maximally activate PKC ζ. PKC ζ may require other proteins to maintain a stable, active conformation, which is lost on *in vitro* analysis. Moreover, PKC ζ activity *in vitro* may reflect the co-precipitation of a phosphatase. It has already been suggested that a calcium activated protein kinase or phosphatase co-purifies with PKC ζ, resulting in its inhibition (Limatola et al., 1994). There is a growing body of evidence indicating that regulation of signalling pathways occurs via protein kinase-phosphatase signalling modules, resulting in direct control of intracellular signalling cascades (Deng et al., 1998; Hu et al., 1998; Westphal et al., 1999). PP2A dephosphorylates associated CaMKIV and so functions as a negative modulator of CaMKIV signalling (Westphal et al., 1998). aPKCs may sequester phosphatases, as has been demonstrated indirectly (Sontag et al., 1997), and in this thesis, aPKCs activation loop site phosphorylation is modulated by PP1/2A phosphatases.

Elucidation of how aPKCs are activated will only come from technical improvements to enable direct visualisation of protein interactions in the cellular context. Already, GFP-tagging, time-lapse, FRET in live cells or microinjection has helped address such problems. To be able to understand the mechanism of activation of PKC ζ, more information can be gleaned from mutation of either phosphorylation sites or using domain structures, if there is an easily identifiable biological endpoint. Studying a component in a pathway which has no apparent physiological activator or role is like hunting for a needle in a haystack. This is not helped by the cross talk and high levels of promiscuity of several kinases in signalling pathways.

7.3 The role of aPKCs in signalling pathways

Atypical PKCs are involved in several signalling pathways, a few will be discussed in detail below. Atypical PKCs have been reported to be activated by EGF, PDGF (Akimoto et al., 1996), insulin (Mendez et al., 1997; Sweeney et al., 1998), and PC-PLC (van Dijk et al., 1997; van Dijk et al., 1997), amongst others, suggesting a linkage between phospholipid turnover and aPKC activation. The discovery of PDK1 has certainly helped by positioning PKC ζ downstream of PI3K and clarified the regulation of aPKCs by $PI(3,4,5)P_3$ lipids. The studies presented in this thesis has set a president for PDK mediated activation of the other PKCs. It is possible that PKC ζ binds

PI3K directly (Carpenter et al., 1993; Gomez et al., 1995), which has been reported for PKC δ and interaction is inhibited by wortmannin (Ettinger et al., 1996). Any PDK1 recruited to the same area by $PI(3,4,5)P_3$ can then phosphorylate PKC ζ. It will be interesting to determine the effects of dominant negative PI3K mutants or expression of PTEN (Maehama and Dixon, 1998) on the phosphorylation of the activation loop of PKC ζ.

PDK1 can directly associate with PKC ζ, as has been shown in chapter 5. It is not known if other proteins are also complexed. The organisation and regulation of several signalling cascades has been shown to occur via scaffold proteins, for example, this has been recently identified in the MAPK cascade (Schaeffer et al., 1998; Whitmarsh et al., 1998) or in NFκB phosphorylation (Cohen et al., 1998; Rothwarf et al., 1998). PDZ domain containing proteins have been implicated in assembling proteins (named after post-synaptic density protein, PSD95, Drosophila discs large *dlg* and ZO1 (Cho et al., 1992; Ponting et al., 1997; Woods and Bryant, 1993). Ina D in *Drosophila* is a prime example where components of the phototransduction cascade, including light-activated ion channels, PLC β and PKC are all colocalised (Tsunoda et al., 1997). Scaffolds or the formation of signalsomes (Mercurio et al., 1999) may help solve spatial-temporal signalling problems and enable prior co-ordination of signalling components, permitting rapid responses to quick, short-lived stimuli. A kinase-anchoring protein, AKAP 79 (Carr et al., 1992) serves as a multivalent scaffold protein targeting PKA, PKC and calcineurin (Klauck et al., 1996) to the membrane. Since PDK1 also phosphorylates PKA, it may be that AKAPs are the scaffold to co-ordinate all these signalling events.

7.3.1 The MAPK cascade

Kinase deficient forms of PKC ζ prevent small T antigen dependent growth in NIH 3T3 fibroblasts by modulation of the mitogen activated protein kinase, MAPK cascade. The involvement of PKCs in the MAPK cascade was first suggested due to sensitivity of the pathway to TPA (Hoshi et al., 1989; Rapp, 1991; Ray and Sturgill, 1987; Rossomando et al., 1989). The MAPK cascade has been implicated in cell proliferation (induced by growth factors and other stress pathways) and control of differentiation (Mischak et al., 1993; Murray et al., 1993; Traverse et al., 1992). Translocation of activated MAPK to the nucleus enables phosphorylation of target molecules, for example, transcription factors activating gene expression (Herskowitz, 1995; Ip and Davis, 1998; Marshall, 1994). MAPK (p42) is a dual specificity kinase requiring both threonine and tyrosine phosphorylation. Two sites are

phosphorylated in the activation loop site by the upstream kinase, MEK1 or MEK2 (Payne et al., 1991). The MAPK kinases are regulated by Raf (Alessi et al., 1994). Raf is modulated partly by association with GTP-Ras and also phosphorylation (Kyriakis et al., 1992; Wu et al., 1993). The regulation of Raf is not so straightforward and several inputs have to be considered, including dimerisation (Luo et al., 1996) or association with other proteins regulating its function, e.g. 14-3-3 (Freed et al., 1994). The role PKCs play in the MAPK cascade is somewhat unsure. There are several PKC phosphorylation sites on Raf (S499, S259, phosphorylated *in vitro* by PKC and *in vivo* in response to TPA (Kolch et al., 1993). Evidence suggests that these sites are not involved in Raf activation. PKCs may affect the MAPK cascade by assisting in membrane-association of Raf or even phosphorylation induced downregulation of Raf. Different PKC isotypes feed into the pathway at different levels (Schoenwasser et al., 1998; Ueda et al., 1996).

The role PKC ζ plays in this cascade and mitogenic activation is unclear. PKC ζ has been implicated in mitogenic signalling due to direct interaction with Ras (Berra et al., 1993; Diaz-Meco et al., 1994). However, no further evidence has substantiated PKC ζ's role in Ras transformation and activation. In NIH 3T3 cells stimulated by Ras activating growth factors or transformed with Ras, PKC ζ had no synergistic effect on the growth properties of cells (Crespo et al., 1995; Montaner et al., 1995). In fact, the opposite effects have been seen where PKC ζ reverted transformation by the v-raf oncogene (Kieser et al., 1996). Overexpression of PKC ζ in prostate cells inhibited metastasis (Powell et al., 1996) but PKC ζ has been implicated in playing a role in IL2 mediated cell proliferation (Gomez et al., 1995).

Subsequently, the role of PKC ζ in the MAPK cascade has been found to be independent of Ras (Berra et al., 1993; Schoenwasser et al., 1998; Ueda et al., 1996) but also activated in a DAG-dependent manner (van Dijk et al., 1997) or by PDGF (van Dijk et al., 1997). These results together with cotransfection and *in vitro* analysis demonstrate that PKC ζ activation of the MAPK cascade occurs in a Raf-independent manner at the level of MEK (Schoenwasser et al., 1998). This activation was found to be indirect and the kinase activity involved was termed ZAK (PKC ζ activated kinase, D. Schoenwasser thesis) but has not been isolated. Whether ZAK is in fact a DAG-dependent protein or PKC ζ activating the MAPK cascade together with other PKCs (Kim et al., 1997) is unknown. Any other MEK activating proteins may be ZAK. Candidates include a novel adipocyte insulin stimulated MEK kinase (I-MEKK, (Haystead et al., 1994) or MKK2 (Wu et al., 1993). PKC ζ has

also been suggested to be not only independent of Raf but also in replacing Raf to mediate Ras induced activation of MEK (Liao et al., 1997). Thus PKC ζ action depends on which pathways are activated. aPKCs *per se* are not oncogenic or tumour supressors, thus their regulation of cell proliferation or growth is more subtle. It will be crucial to determine pathways aPKCs lie on, possibly using a knockout approach or by characterising interacting proteins and resulting cellular localisation. Regulation of aPKCs may require more acute stimuli, for example, oxidative stresses (Waite et al., 1997) or viral infection.

7.3.2 Nuclear signalling

PKC ζ can be found associated with spindle fibres (even though this does not imply nuclear localisation since the nuclear envelope is disassembled at this point (Lehrich and Forrest, 1994); stimulate gene expression of integrins and phosphorylate ribonuclear proteins, for example nucleolin (Zhou et al., 1997). All these observations question whether PKC ζ directly plays an active role in nuclear signalling or if all these effects are indirect. Data presented in chapter 6 shows nuclear localisation of the PKC ζ Vo domain. How this occurs is still unclear. Data in chapter 6 also suggests that nucleolin (a nuclear-cytoplasmic shuttling protein) may interact with PKC ζ. Thus PKC ζ may piggy-back this protein to become localised in the nucleus. In virally transfected cells, PKC ζ may bind small or large T antigens and become imported into the nucleus (Sontag et al., 1997). Alternatively, if PKC ζ can sequester phosphatases, this may enable nuclear localisation i.e. with PNUTs (Allen et al., 1998). PKC ζ was found to interact with hnRNPA1 (Municio 1995), which is a nucleocytoplasmic shuttling protein. Expression of constitutively active PKC ζ stimulated RNPA1 shuttling and reduced cytosolic levels of this protein. What happens to PKC ζ localisation under such conditions was not discussed. An alternative mechanism of nuclear localisation may arise due to small ubiquitin-like modifiers, SUMO, coating of PKC ζ, as has been seen for RanGAP1 nuclear uptake (Mahajan et al., 1998; Matunis et al., 1996; Matunis et al., 1998). Phosphorylation of the activation loop of ERK2 (Khokhlatchev et al., 1998) has been found to be sufficient for homodimerisation (and exposure of a NLS) and nuclear localisation, independent of kinase activity. This is not the case for PKC ζ since activation by either deleting the PSS region or using phosphorylation site mutants did not result in nuclear localisation. Thus it is likely that nuclear localisation is not an intrinsic property of PKC ζ and is linked to cell-cycle or agonist-stimulated events.

The role of PKC ζ in the nucleus is unknown. However, PKC ζ phosphorylates several nucleoproteins (for example, nucleolin). Phosphorylation of hnRNPA1 inhibits its capacity to bind single-stranded oligonucleotides. It will be interesting to determine where in the cell PKC ζ phosphorylates the ribonuclear proteins, if this is a cell cycle dependent event, or what role PKC ζ plays in RNA binding. Zinc finger proteins have been implicated in binding RNA directly (Grondin et al., 1996). Potentially PKC ζ may be on a general pathway or act in a similar manner to other proteins associated with RNA binding proteins, e.g. Vav, which can activate gene expression (Hobert et al., 1994).

PKC ζ was hypothesised to be the kinase involved in phosphorylation of IκB, enabling release of NFκB and entry into the nucleus to act as a transcription factor, and so PKC ζ indirectly affects NF-κB gene activation (Diaz et al., 1991; Diaz-Meco et al., 1994; Diaz-Meco et al., 1993). Recent work has identified the kinase involved (IκK α and β (Malinin et al., 1997; Mercurio et al., 1997) but does not rule out the possibility that PKC ζ acts upstream and regulates the IκK. The zinc finger of PKC ζ binds to a leucine zipper motif of par4 (Diaz-Meco et al., 1996). This region was also found in the C-terminal region of death domains, for example found in FADD, TRADD or TNFR1. Thus PKC ζ may be able to interact with TRADD or TNFR1 (Hsu et al., 1996; Hsu et al., 1995). It is also possible a third pathway of NFκB dependent transcriptional activation exists which is independent of the TRAF/NIK/IKK pathway. PKC ζ has been found to be activated by both TNF and IL1 (Rzymkiewicz et al., 1996) pathways, resulting in NFκB activation, thus perhaps PKC ζ plays an upstream role in NFκB-mediated gene activation. However, since PKC ζ can enter the nucleus, it may be able to mediate transcription directly.

7.3.3 PKC ζ and Viruses

PKC ζ is able to phosphorylate the P protein (involved in transactivation of viral RNA polymerases) of human parainfluenza virus type 3 (HPIV) (De et al., 1995). Molecular parasitology may give PKC ζ an important role in the viral life cycle, in particular for the paramyxovirus family (negative strand RNA viruses). PKC ζ PSS peptides resulted in a dose-dependent decrease in accumulation of Sendai viral progeny (Huntley et al., 1997) and PKC ζ mediated NF-κB induced activation responsible for persistent HIV-1 infection in monocytes (Folgueira et al., 1996). PKC ζ has also been found packaged into several virions. Viruses may give insight into how PKC ζ is activated. A

specific viral envelope protein or lipid may be required for activation. Several cellular kinases play vital roles in viral gene expression, which may implicate PKC ζ working in a co-operative manner, i.e. with PKA (Cobianchi et al., 1993) or casein kinase II (Das et al., 1995). PKC ζ appears to regulate viral replication of respiratory viruses, indicating that specific cellular localisations of kinases may control the role of host kinases in viral replication. Viruses replicate in the cytoplasm, however, if PKC ζ is packaged in the virion, it may readily change its cellular or tissue distribution. Understanding how viruses hijack cellular signalling pathways and utilise host signalling components will be important in generating drug therapies.

7.3.4 Regulation of AGC family members

Data presented in chapter 4 utilising a partly-activated mutant (EE), demonstrates that PKC ζ can cause an increase in the FSY site phosphorylation of PKC δ. Moreover, PKC ζ does associate with PKC α and PKC δ in a TPA-dependent manner. It is not clear at present if PKC ζ is the FSY site kinase directly. PKC ζ may bind and protect the V5 region from the action of phosphatases. Whether PKC ζ can mediate FSY site phosphorylation *in vitro* has yet to be determined. Several candidates have been identified as FSY site kinases, for example, ILK (Delcommenne et al., 1998). It will be interesting to identify the direct physiological kinase. The FSY site may be an autophosphorylation site for the cPKCs (Keranen et al., 1995) or phosphorylation may occur by PKC homodimerisation. Alternatively, several different stimuli may result in phosphorylation of this site by several different kinases. In light of the conservation of phosphorylation events in the activation loop site by PDK1, it is possible that there is one unique kinase which phosphorylates all AGC kinase family members. aPKCs have been found to bind to PKB (Konishi et al., 1994; Konishi et al., 1994) and $p70^{S6K}$ (Akimoto et al., 1998) but whether this correlates with FSY site phosphorylation has yet to be determined.

7.4 PKC ζ and the cytoskeleton

What role does PKC ζ play in cytoskeletal architecture? Data presented in chapter 4 and chapter 6 suggests that activated forms of PKC ζ result in membrane ruffling and pseudopodia formation. Some groups find PKC ζ associated with actin (Gomez et al., 1995) or tubulin (Garcia-Rocha et al., 1997) but not both. How this correlates with the role of PKC ζ in mitosis or other cytoskeletal-mediated events is unclear. PKC ζ has also been

implicated in playing a role in cell adhesion, by specifically affecting tight junction sorting. PKC ζ may act as the kinase mediating cellular stimulation and causing cell adhesion or movement. PKC ζ has been found to mediate integrin responses, but this only appears to be on the level of gene activation (Xu and Clark, 1997; Xu et al., 1996). PKC ζ has been found localised to cell-cell contacts and not at free edges of cells. By immunofluorescence data, PKC ζ was localised to tight junctions and co-localised with a zona occludens protein (ZO1) (Mitic and Anderson, 1998). PKC ζ can phosphorylate ZO1 *in vitro* (Stuart and Nigam, 1995). This localisation is also associated with G protein localisation (Gα 12) (Dodane and Kachar, 1996). What role PKC ζ plays in either tight junction sorting or regulation of tight junction permeability is unknown. PKC ζ may bind to and mediate transport of ZO1, which acts as a scaffold to organise occludin proteins at tight junctions. Data for PKC3 in *C.elegans* demonstrates that *in vitro*, PKC3 can bind a PDZ containing protein, the *C.elegans* equivalent of PICK 1. Binding can occur via the DXV C-terminus. Potentially, since ZO1 has a PDZ domain, this binding site may explain the interaction with PKC ζ. If this is a V5 domain interacting-protein is unknown. It would be interesting to determine what could bind to the PKC ζ Ψ I since this has a novel C-terminus and if the C-terminus affected cellular localisation, compared to wild-type PKC ζ. However, the discovery of the novel aPKC binding protein, ASIP, gives insight into this issue. ASIP colocalised with PKC λ to tight junctions (Izumi et al., 1998). Interestingly, PKC λ does not bind to the PDZ domain, thus ASIP may bring PKC into contact with other signalling components capable of binding the PDZ domains, for example PKC α. It is unclear if ASIP is the same protein as the *C.elegans* PICK 1 (Wu et al., 1998). In tight junctions, colocalisation of ASIP with ZO1 occurs which clarifies previous data. However, it is still unresolved what role PKC ζ plays in this location.

Under several circumstances, PKC α and PKC ζ are colocalised to similar cell compartments. For example, PKC ζ has also been found localised in caveolae (Oka et al., 1997). PKC α is also localised there in a TPA-dependent manner (Mineo et al., 1998; Smart et al., 1994). Caveolae are vesicular invaginations of the plasma membrane (Okamoto et al., 1998) and may play a role in generating preassembled signalling complexes. Signalling pathways are intact within caveolae and active, for example, PDGF activates the MAPK cascade in caveolae (Liu et al., 1997). PKC α is associated with cell contacts and tight junctions. In Hep G2 cells, both PKC α and PKC ζ could be associated with the transgolgi network (Westermann et al., 1996). PKC ζ is associated with p62 in the endosomal pathway and PKC α and PKC

ζ are found together in the early endosomal compartment (C.Prevostel in preparation). Together with the data presented in chapter 4 for the colocalisation of PKC α and PKC ζ, what role does this association have in the regulation of cellular events? Do these two proteins activate each other or interact to target each other to specific cellular locations and signalling pathways? Several processes that are inhibited by classical PKC inhibitors may be mediated by PKC ζ, suggesting a role of c/nPKCs in the functioning of PKC ζ. For example, in tight junctions, transepithelial electrical resistance (TER) is inhibited by calphostin C (Stuart and Nigam, 1995). Moreover, in ischemia-reperfusion of rat heart, where there is massive calcium influx, PKC ζ is activated and both PKC α and PKC ζ are translocated to the nucleus. The interrelationships and control of PKC family members by other PKCs may no longer be a joke (Newton and Taylor, 1995). It will be intriguing to see how the distinct roles of all the PKC family members, specifically the aPKCs, can be unravelled.

It will be interesting to pinpoint the role of aPKCs in integrin and vesicle trafficking, cytoskeletal changes, cell polarity, mitosis and anoikis. In summary, we have clearly established PKC ζ (and other PKCs) downstream of PDK1 on PI3K pathways. Yet how aPKCs mediate other signalling pathways has still to be resolved (Figure 7.1).

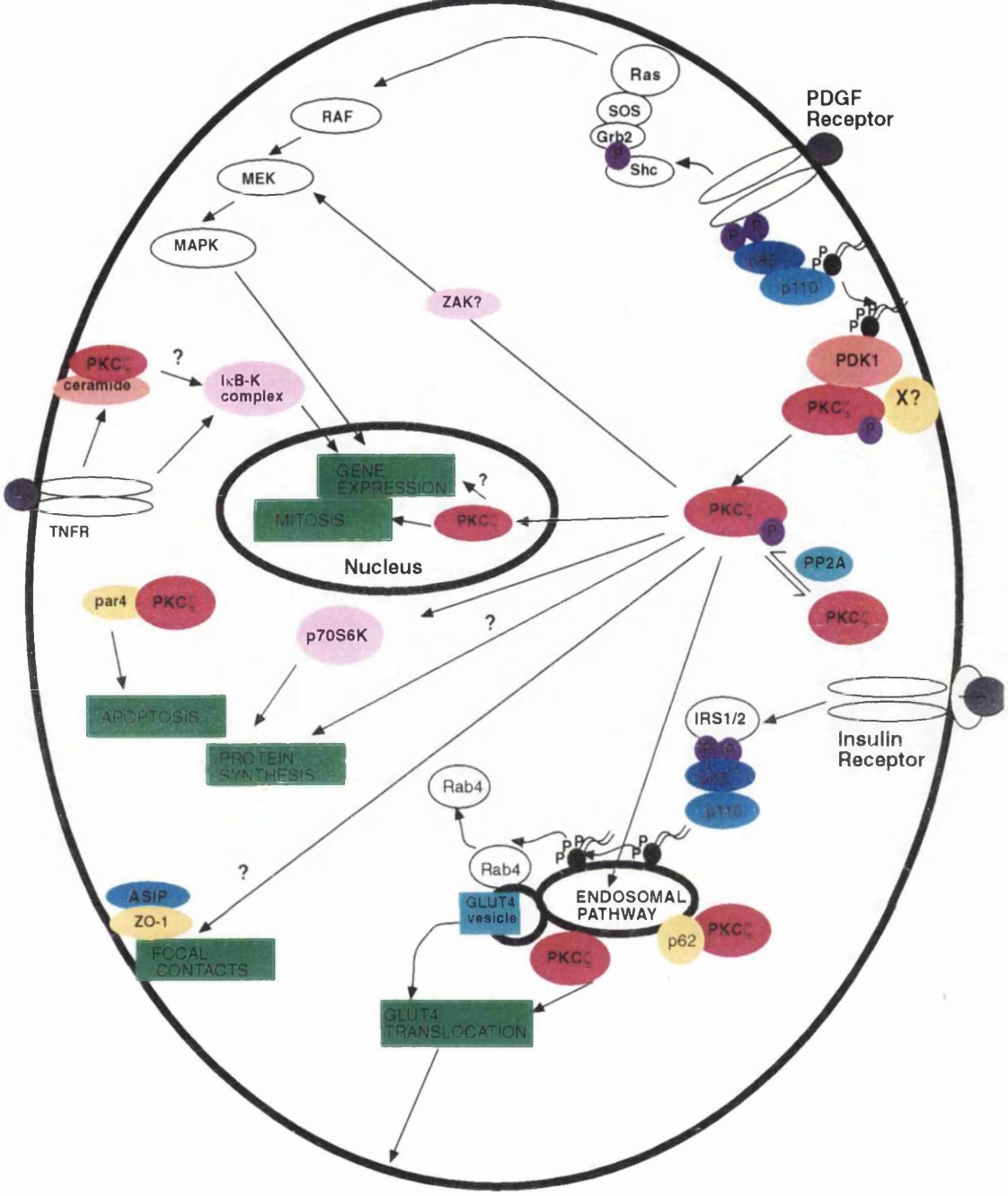


Fig 7.1
The Roles of PKC ζ in Cellular Signalling

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